UTILITY PATENT APPLICATION TRANSMITTAL (Small Entity) *(Only for new nonprovisional applications under 37 CFR 1.53(b))*	Docket No. 004.00191
	Total Pages in this Submission

TO THE ASSISTANT COMMISSIONER FOR PATENTS
Box Patent Application
Washington, D.C. 20231

Transmitted herewith for filing under 35 U.S.C. 111(a) and 37 C.F.R. 1.53(b) is a new utility patent application for an invention entitled:

T-TYPE CALCIUM CHANNEL

and invented by:

Ming Li

If a CONTINUATION APPLICATION, *check appropriate box and supply the requisite information:*

☐ **Continuation**　☐ **Divisional**　☐ **Continuation-in-part (CIP)**　of prior application No.: _____

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Enclosed are:

Application Elements

1. ☐ Filing fee as calculated and transmitted as described below

2. ☒ Specification having _____86_____ pages and including the following:

　a. ☒ Descriptive Title of the Invention

　b. ☒ Cross References to Related Applications *(if applicable)*

　c. ☒ Statement Regarding Federally-sponsored Research/Development *(if applicable)*

　d. ☐ Reference to Microfiche Appendix *(if applicable)*

　e. ☒ Background of the Invention

　f. ☒ Brief Summary of the Invention

　g. ☒ Brief Description of the Drawings *(if drawings filed)*

　h. ☒ Detailed Description

　i. ☒ Claim(s) as Classified Below

　j. ☒ Abstract of the Disclosure

P01USML/REV03

Application Elements (Continued)

3. ☒ Drawing(s) *(when necessary as prescribed by 35 USC 113)*

    a. ☒ Formal   b. ☐ Informal   Number of Sheets   15

4. ☐ Oath or Declaration

    a. ☐ Newly executed *(original or copy)*   ☐ Unexecuted

    b. ☐ Copy from a prior application (37 CFR 1.63(d)) *(for continuation/divisional application only)*

    c. ☐ With Power of Attorney   ☐ Without Power of Attorney

    d. ☐ *DELETION OF INVENTOR(S)*
       Signed statement attached deleting inventor(s) named in the prior application,
       see 37 C.F.R. 1.63(d)(2) and 1.33(b).

5. ☐ Incorporation By Reference *(usable if Box 4b is checked)*
    The entire disclosure of the prior application, from which a copy of the oath or declaration is supplied under Box 4b, is considered as being part of the disclosure of the accompanying application and is hereby incorporated by reference therein.

6. ☐ Computer Program in Microfiche

7. ☒ Genetic Sequence Submission *(if applicable, all must be included)*

    a. ☒ Paper Copy

    b. ☐ Computer Readable Copy

    c. ☐ Statement Verifying Identical Paper and Computer Readable Copy

Accompanying Application Parts

8. ☐ Assignment Papers *(cover sheet & documents)*

9. ☐ 37 CFR 3.73(b) Statement *(when there is an assignee)*

10. ☐ English Translation Document *(if applicable)*

11. ☐ Information Disclosure Statement/PTO-1449   ☐ Copies of IDS Citations

12. ☐ Preliminary Amendment

13. ☒ Acknowledgment postcard

14. ☒ Certificate of Mailing

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	Docket No.
	004.00191

UTILITY PATENT APPLICATION TRANSMITTAL
(Small Entity)
(Only for new nonprovisional applications under 37 CFR 1.53(b))

Total Pages in this Submission

Accompanying Application Parts (Continued)

15. ☐ Certified Copy of Priority Document(s) *(if foreign priority is claimed)*

16. ☐ Small Entity Statement(s) - Specify Number of Statements Submitted: _____

17. ☐ Additional Enclosures *(please identify below):*

Fee Calculation and Transmittal

CLAIMS AS FILED

For	#Filed	#Allowed	#Extra	Rate	Fee
Total Claims	60	- 20 =	40	x $9.00	$360.00
Indep. Claims	13	- 3' =	10	x $39.00	$390.00
Multiple Dependent Claims (check if applicable) ☐					$0.00
				BASIC FEE	$380.00
OTHER FEE *(specify purpose)* _____					$0.00
				TOTAL FILING FEE	$1,130.00

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    ☐ Charge the issue fee set in 37 C.F.R. 1.18 at the mailing of the Notice of Allowance, pursuant to 37 C.F.R. 1.311(b).

Dated: **August 26, 1999**

_____
          Signature

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P01USML/REV03

CERTIFICATE OF MAILING BY "EXPRESS MAIL" (37 CFR 1.10)	Docket No.
Applicant(s): Ming Li	004.00191

Serial No. To Be Assigned	Filing Date Herewith	Examiner Unknown	Group Art Unit Unknown

Invention:

T-TYPE CALCIUM CHANNEL

I hereby certify that this   <u>**Utility Patent Application Transmittal (3 pages)**</u>

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Applicant(s):   Ming Li

Serial No. To Be Assigned	Filing Date Herewith	Examiner Unknown	Group Art Unit Unknown

Invention:

T-TYPE CALCIUM CHANNEL

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P06A/REV02

CERTIFICATE OF MAILING BY "EXPRESS MAIL" (37 CFR 1.10)

Applicant(s): Ming Li

Docket No.	004.00191

Serial No.	Filing Date	Examiner	Group Art Unit
To Be Assigned	**Herewith**	**Unknown**	**Unknown**

Invention:

T-TYPE CALCIUM CHANNEL

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P06A/REV02

TITLE: T-TYPE CALCIUM CHANNEL

INVENTOR: Ming Li

DOCKET NO.:004.00191

T-TYPE CALCIUM CHANNEL

This application claims priority of U.S. Provisional Patent Application No. 60/098,004, filed August 26, 1998,
5 and of U.S. Provisional Patent Application No. 60/117,399, filed January 27, 1999.

FIELD OF THE INVENTION

The present invention relates generally to calcium
15 channel proteins, and more particularly to pancreatic T-type calcium channel proteins and uses thereof.

BACKGROUND OF THE INVENTION

Throughout this application various publications are
20 referenced, many in parenthesis. Full citations for each of these publications are provided at the end of the Detailed Description. The disclosures of each of these publications in their entireties are hereby incorporated by reference in this application.

25 Insulin secretion from pancreatic β-cells is the primary physiological mechanism of blood glucose regulation. A rise in blood glucose concentration stimulates release of insulin from the pancreas, which in turn promotes glucose uptake in peripheral tissues and
30 consequently lowers blood glucose levels, reestablishing euglycemia. Non-insulin dependent diabetes mellitus (NIDDM)(type II diabetes) is associated with an impairment in glucose-induced insulin secretion in pancreatic β-cells (Vague and Moulin, 1982).

35 Voltage-gated Ca^{2+} channels mediate a rapidly activated inward movement of Ca^{2+} ions that underlies the

stimulation of insulin secretion in β-cells (Boyd III
1991). In different tissues, four types of Ca^{2+} channels
have been described (L(P/Q), T, N, and E channels). The
purified L-type Ca^{2+} channel consists of five subunits: α_1,
5 α_2, β, γ, δ (Catterall 1991). The primary structure of
the α_1 subunit is organized in four homologous domains
containing six transmembrane segments (Catterall 1988).

Rat and human pancreatic β-cells are equipped with
L-type and T-type Ca^{2+} channels (Hiriart and Matteson,
10 1988; Davalli et al., 1996). L-type Ca^{2+} channels,
activated at high voltages and having large unitary
conductance and dihydropyridine-sensitivity, are
considered the major pipeline for Ca^{2+} influx into the β-
cell (Keahey et al., 1989). In contrast, T-type calcium
15 channels activate at low voltages and have small unitary
conductance and dihydropyridine-insensitivity.

The physiological function of T-type Ca^{2+} channels in
β-cell insulin-secretion has been demonstrated
(Bhattacharjee et al., 1997). These channels facilitate
20 exocytosis by enhancing electrical activity in these
cells. L-type and T-type Ca^{2+} channels, under normal
conditions, work in concert promoting the rise in $[Ca^{2+}]_i$
during glucose-stimulated insulin secretion. In β-cells,
over-expressed T-type Ca^{2+} channels may be, at least in
25 part, responsible for the hyper-responsiveness of insulin
secretion to non-glucose depolarizing stimuli in GK rat
and in rat with NIDDM induced by neonatal injection of
streptozotocin (Kato et al., 1994; Kato et al., 1996).
However, over-expressed T-type calcium channels over time
30 will ultimately lead to an elevation of basal Ca^{2+} through
it's window current properties. Therefore, there is a
dual effect of T-type Ca^{2+} channels in β-cells depending
upon channel number and membrane potential.

Two isoforms of L-type Ca^{2+} channel α1 subunits have been identified in β-cells (Seino et al., 1992; Yaney et al., 1992). The rat neuronal T-type calcium channel has recently been cloned (Perez-Reyes et al., 1998). Other

5 subunits of T-type Ca^{2+} channel have yet to be identified.

Given the evidence that T-type calcium channels are associated with type II diabetes, a need exists to further characterize T-type calcium channels.

10   **SUMMARY OF THE INVENTION**

To this end, the subject invention provides an isolated nucleic acid molecule encoding a pancreatic T-type calcium channel. The invention also provides an antisense nucleic acid molecule complementary to at least

15 a portion of the mRNA encoding the pancreatic T-type calcium channel.

The isolated nucleic acid molecules of the invention can be inserted into suitable expression vectors and/or host cells. Expression of the nucleic acid molecules

20 encoding the pancreatic T-type calcium channel results in production of pancreatic T-type calcium channel in a host cell. Expression of the antisense nucleic acid molecules in a host cell results in decreased expression of the pancreatic T-type calcium channel.

25 The invention further provides a ribozyme having a recognition sequence complementary to a portion of mRNA encoding a pancreatic T-type calcium channel. The ribozyme can be introduced into a cell to also achieve decreased expression of pancreatic T-type calcium channel

30 in the cell.

The invention further provides a method of screening a substance for the ability of the substance to modify T-type calcium channel function, and a method of obtaining DNA encoding a pancreatic T-type calcium channel.

Further provided is an isolated nucleic acid molecule encoding a pancreatic T-type calcium channel, wherein the nucleic acid molecule encodes a first amino acid sequence having at least 90% amino acid identity to

5 a second amino acid sequence. The second amino acid sequence is as shown in SEQ ID NO:2.

The invention further provides a DNA oligomer capable of hybridizing to a nucleic acid molecule encoding a pancreatic T-type calcium channel. The DNA

10 oligomer can be used in a method of detecting presence of a pancreatic T-type calcium channel in a sample, which method is also provided by the subject invention.

The invention also provides an isolated pancreatic T-type calcium channel protein, and antibodies or

15 antibody fragments specific for the pancreatic T-type calcium channel protein. The antibodies and antibody fragments can be used to detect the presence of the pancreatic T-type calcium channel protein in samples. Further provided is an isolated pancreatic T-type calcium

20 channel protein encoded by a first amino acid sequence having at least 90% amino acid identity to a second amino acid sequence, the second amino acid sequence as shown in SEQ ID NO:2.

The subject invention further provides a method of

25 modifying insulin secretion by pancreatic beta cells, the method comprising modifying levels of functional T type calcium channels in the pancreatic beta cells. The invention further provides a method of treating type II diabetes in a subject, the method comprising

30 administering to the subject an amount of a compound effective to modify levels of functional T type calcium channel in the pancreatic beta cells of the subject.

The invention also provides a method of modifying basal calcium levels in cells, a method of modifying the

action potential of L type calcium channels in cells, a
method of modifying pancreatic beta cell death, a method
of modifying pancreatic beta cell proliferation, and a
method of modifying calcium influx through L type calcium
5   channels in cells, each of the methods comprising
modifying levels of functional T type calcium channels in
the cells.

BRIEF DESCRIPTION OF THE DRAWINGS

10   These and other features and advantages of this
invention will be evident from the following detailed
description of preferred embodiments when read in
conjunction with the accompanying drawings in which:

Fig. 1A illustrates a comparison of the nucleotide
15 sequence of α_1G-INS (1) and α_1G (2) at the 5'-end regions
(aa1-67 of α_1G). The four insertions are indicated with
arrow heads. The capital ATG represents the start codon
for each cDNA;

Fig. 1B is a schematic illustration representing
20 partial rat genomic nucleotide composition between Domain
III and IV. Genomic DNA contained an exon specific to α_1G
(shaded circle) and an exon specific to the α_1 subunit of
T-type Ca^{2+} deduced from INS-1 (shaded rectangle) between
4845 and 5256 of the cDNA sequence. Other exons (open
25 rectangles) are identical between the two cDNAs. The bold
letters indicate the nucleotides coding Gly-1667;

Figs. 2A-2D illustrate expression of α_1G-INS in
Xenopus oocytes. Fig. 2A illustrates 40 mM Ca^{2+} currents
elicited by depolarizing pulses from - 60 to 40 mV. Fig.
30 2B illustrates time constants of activation and
inactivation measured at test potentials between -30 and
30 mV. The time constants of activation were obtained by
fitting the increasing portion (activation) of currents
with the Hodgkin-Huxley equation where the m value was

designated as four (n = 6). The time constants of
inactivation were obtained by single exponential fitting
(n = 6). Fig. 2C illustrates voltage-dependent
conductance (n = 7) and Fig. 2D illustrates steady-state
5 inactivation (n = 3) of expressed currents in oocytes.
The holding potential for Figs. 2C and 2D was -80 mV. The
currents in Fig. 2D were measured at -10 mV after varying
1000 ms pre-pulse potentials. Peak currents were
normalized to the maximum current and then averaged
10 (error bars represent SE);

Figs. 3A and 3B illustrate accumulative dose
response relationships of the inhibitory effects of
mibefradil on T- and L-type Ca^{2+} currents. Currents were
measured with the whole-cell patch clamp configuration.
15 Data from four experiments were normalized individually
and than plotted as mean $\pm$ standard error. Fig. 3A
illustrates curve which was generated by fitting the data
using one-to-one binding curve according to the equation
$1/(1 + [mibefradil]/K_d)$. Fig. 3B is a dose response of
20 L-type Ca^{2+} current obtained when perfusion of solutions
containing different concentrations of mibefradil;

Fig. 4 illustrates reversibility of the inhibition
of T and L-type currents by $NiCl_2$ and mibefradil,
respectively. Open and solid circles represent the T-type
25 Ca^{2+} current recorded before and after $NiCl_2$ (2 μl of 30
μM) and mibefradil (2 μl of 10 μM) were administrated,
respectively. The open squares represent the L-type Ca^{2+}
current recorded before and after mibefradil (2 μl of 10
μM) was administrated with perforated patch clamp
30 configuration. The T-type Ca^{2+} current was measured at -30
mV with a holding potential of -80 mV with whole cell
configuration. Arrow indicates the time when the drugs
were delivered. n =3 for each group experiments;

Figs. 5A and 5B illustrate the long-term effect of mibefradil (10 nM) on L- and T- Ca^{2+} currents in the perforated-patch configuration. In Fig. 5A, solid and open circles represent the L-type Ca^{2+} current recorded in

5   the cells with and without administration of mibefradil, respectively. Solid triangles represent T-type Ca^{2+} currents recorded in the cells after administrating mibefradil. Mibefradil were delivered at time zero. n = 4 for each group experiments. In Fig. 5B, cells were

10   cultured in medium with or without co-incubating 10 nM mibefradil for 2 hours. The current densities were recorded with perforated patch clamp configuration. n = 14 for each group experiments;

Fig. 6A illustrates accumulation of dm-mibefradil in

15   the cells measured with mass spectrometry. The cells were first incubated with mibefradil (20 μM) for the duration indicated on the figure (n = 3). The inset (Fig. 6B) shows the primary data of mass spectrometry indicating peaks at 496 and 424, which correspond to mibefradil and

20   dm-mibefradil, respectively;

Fig. 7A illustrates the effect of mibefradil and dm-mibefradil on L-type Ca^{2+} currents from inside cells. n = 8, *, p < 0.01 to the control;

Fig. 7B illustrates the effect of mibefradil or

25   dm-mibefradil on T-type Ca^{2+} current from inside cells n = 4. All data were collected at 5 min after formation of whole cell patch. The pipette solution contained 1 μM of drug;

Fig. 8 illustrates basal $[Ca^{2+}]_i$ measured in an INS-1

30   cell. T-type calcium channel antagonist mibefradil (1 μM) reduced basal $[Ca^{2+}]_i$ in a single cell in the bath solution without glucose. The $[Ca^{2+}]_i$ was measured with the emission ratio of Fura-2 AM (F380/F340) then

calibrated with the standard solution purchased from
Molecular Probes Inc. (OR);

Fig. 9A illustrates that intracellular perfusion of
a solution containing 272 nM free calcium concentration

5   inhibits the L-type calcium current. Currents were
elicited by a step voltage to +10 mV, with holding
potential of -80 mV;

Fig. 9B illustrates the effect of perfusing in high
calcium concentration on the IV calcium current

10 relationship. Closed circles represent the cell before
perfusion, and open circles represent perfusion of 272 nM
free calcium;

Fig. 9C illustrates the effect of intracellular
perfusion of different calcium concentrations on L-type

15 calcium current over time. Squares represent perfusion
from high calcium to low calcium (intracellular solution
contained 632 nM then perfused by a solution with 10 mM
EGTA), triangles represent perfusion from low calcium to
272 nM calcium, and circles represent low calcium to 632

20 nM calcium;

Fig. 9D illustrates the effect of high calcium on
the T-type calcium channel current. Tail currents were
elicited by a voltage step to -30 mV for 10 ms;

Fig. 10 illustrates that reestablishment of basal

25 calcium causes stereotyped calcium influx. A cell was
twice perfused with 50 mM KCl with an intervening
perfusion of the original bath solution to restore
membrane potential;

Fig. 11 illustrates that elevated basal Ca^{2+} causes a

30 defect in the Ca^{2+} transient. A cell was twice perfused
with 50 mM KCl with an intervening perfusion of the
original bath solution to restore membrane potential.
The second perfusion occurred prior to reestablishment of
the original basal $[Ca^{2+}]_i$ of about 60 nM;

Fig. 12 illustrates a model for glucose-stimulated insulin release;

Fig. 13 illustrates that mibefradil (1 μM) blocks T- and L-type Ca^{2+} current in INS-1 cells. The relative current of T type Ca channel is obtained by measuring their slow deactivated tail current (n = 8);

Fig. 14 illustrates that mibefradil and $NiCl_2$ reversibly block T type Ca^{2+} current in INS-1 cells. Drugs were administered into the recording chamber at 180 seconds from the beginning of recording. N = 3;

Fig. 15 illustrates the activation and inactivation curves for INS-1 cells, revealing a "window current";

Fig. 16 illustrates the effect of $NiCl_2$, mibefradil, and nifedipine on basal insulin secretion in NIT-1 cells. The glucose concentration is 3 mM in the experiments;

Fig. 17 illustrates that the T type calcium channel antagonist $NiCl_2$ (30 μM) reduced the frequency of transient spontaneous elevation of $[Ca^{2+}]_i$, in a single cell in the bath solution without glucose;

Fig. 18 illustrates the effect of 30 mM $NiCl_2$ on the $[Ca^{2+}]_i$ under non-stimulus conditions. Data was collected from the cells with "high" initial basal $[Ca^{2+}]_i$ (about 100 nM). n = 13;

Figs. 19A and 19B illustrate that hyperpolarization induced an increase in number of action potentials and a decrease in onset latencies. N = 40;

Figs. 20A and 20B illustrate the dose-dependent effect of $NiCl_2$ on insulin secretion. Cells were placed in a medium containing 11.1 mM glucose and a decrease in onset latencies. N = 40;

Fig. 21 illustrates "run-up" in whole cell recording;

Fig. 22 illustrates KCl induced Ca^{2+} influx in the INS-1 cells treated with streptozotocin. n = 13;

Fig. 23A-23F illustrate the results of cytokine treatment. LVA Ca^{2+} currents were induced by cytokine treatment (IL-1β, 25 U/ml; IFNγ, 300 U/ml) for 6 h in primary cultured mouse islet cells, but not in α-TC1

5   cells. An LVA current was elicited by a -40 mV test pulse in an islet cell (Fig. 23A), but the same current was not detected in α-TC1 cells (Fig. 23C). The Ca^{2+} current density-voltage relationships obtained from islet cells (Fig. 23B) and α-TC1 cells (Fig. 23D) with and

10 without cytokine treatment are shown. The open circles represent the current densities of untreated cells (n = 10 for islet cells; n = 20 for α-TC1 cells), and the filled circles represent the current densities of cells treated by cytokines (n = 21 for islet cells; n = 21 for

15 α-TC1 cells). The recordings were elicited by voltages ranging from -50 to +20 mV for 100 msec. All experiments were performed at -80 mV. Fig. 23E shows steady state inactivation of LVA tail currents elicited by a 10-msec depolarizing (-10 mV) pulse followed by a 50-msec

20 hyperpolarizing pulse (-100 mV), with a holding potential of -80 mV. Fig. 23F shows that $NiCl_2$ (10 μM) blocked the cytokine induced LVA Ca^{2+} current elicited at a -30 mV step pulse in an islet cell;

Figs. 24A and 24B illustrate the effects of

25 cytokines on $[Ca^{2+}]_i$ in mouse islet cells and α-TC1 cells. In Fig. 24A, basal $[Ca^{2+}]_i$ of primary cultured mouse islet cells was approximately 3-fold higher after cytokine treatment. $NiCl_2$ (10 μM), but not nifedipine (10 μM), prevented the increase in $[Ca^{2+}]_i$. In Fig. 24B, basal

30 $[Ca^{2+}]_i$ in α-TC1 cells was unaffected by cytokine treatment. Cytokine treatment consisted of IL-1β (25 U/ml) and IFNγ (300 U/ml) for 6 h; and

Figs. 25A and 25B illustrate the effects of $NiCl_2$ on cytokine-induced β-TC3 cell death. $NiCl_2$ (20 μM)

significantly reduced cell death induced by cytokines in both a time (Fig. 25A) and dose-dependent (Fig. 25B) manner (n = 3). Cytokine treatment consisted of IL-1β (25 U/ml), IFNγ (100 U/ml), and TNFα (100 U/ml) in Fig.

5 25A and of IL-1β (25 U/ml), TNFα (100 U/ml), and various concentrations of IFNγ as indicated in Fig. 25A. The first dose, 0, represents zero concentration for all three cytokines. The concentration of nifedipine was 10 μM in both Fig. 25A and Fig. 25B.

10

DETAILED DESCRIPTION OF THE INVENTION

The term "nucleic acid", as used herein, refers to either DNA or RNA. "Nucleic acid sequence" or "polynucleotide sequence" refers to a single- or

15 double-stranded polymer of deoxyribonucleotide or ribonucleotide bases read from the 5' to the 3' end. It includes both self-replicating plasmids, infectious polymers of DNA or RNA, and nonfunctional DNA or RNA.

"Isolated" nucleic acid refers to nucleic acid which

20 has been separated from an organism in a substantially purified form (i.e. substantially free of other substances originating from that organism), and to synthetic nucleic acid.

By a nucleic acid sequence "homologous to" or

25 "complementary to", it is meant a nucleic acid that selectively hybridizes, duplexes or binds to DNA sequences encoding the protein (channel) or portions thereof when the DNA sequences encoding the protein are present in a human genomic or cDNA library. A DNA

30 sequence which is similar or complementary to a target sequence can include sequences which are shorter or longer than the target sequence so long as they meet the functional test set forth.

Typically, the hybridization is done in a Southern
blot protocol using a 0.2X SSC, 0.1% SDS, 65°C wash. The
term "SSC" refers to a citrate-saline solution of 0.15M
sodium chloride and 20 mM sodium citrate. Solutions are
5   often expressed as multiples or fractions of this
concentration. For example, 6X SSC refers to a solution
having a sodium chloride and sodium citrate concentration
of 6 times this amount or 0.9 M sodium chloride and 120
mM sodium citrate. 0.2X SSC refers to a solution 0.2
10 times the SSC concentration or 0.03M sodium chloride and
4 mM sodium citrate.

The phrase "nucleic acid molecule encoding" refers
to a nucleic acid molecule which directs the expression
of a specific protein or peptide. The nucleic acid
15 sequences include both the DNA strand sequence that is
transcribed into RNA and the RNA sequence that is
translated into protein or peptide. The nucleic acid
molecule includes both the full length nucleic acid
sequences as well as non-full length sequences derived
20 from the full length protein. It being further
understood that the sequence includes the degenerate
codons of the native sequence or sequences which may be
introduced to provide codon preference in a specific host
cell.

25   The term "located upstream" as used herein refers to
linkage of a promoter upstream from a nucleic acid (DNA)
sequence such that the promoter mediates transcription of
the nucleic acid (DNA) sequence.

The term "vector", refers to viral expression
30 systems, autonomous self-replicating circular DNA
(plasmids), and includes both expression and
nonexpression plasmids. Where a recombinant
microorganism or cell is described as hosting an
"expression vector," this includes both extrachromosomal

circular DNA and DNA that has been incorporated into the host chromosome(s). Where a vector is being maintained by a host cell, the vector may either be stably replicated by the cells during mitosis as an autonomous

5 structure, or the vector may be incorporated within the host's genome.

The term "plasmid" refers to an autonomous circular DNA molecule capable of replication in a cell, and includes both the expression and nonexpression types.

10 Where a recombinant microorganism or cell is described as hosting an "expression plasmid", this includes latent viral DNA integrated into the host chromosome(s). Where a plasmid is being maintained by a host cell, the plasmid is either being stably replicated by the cell during

15 mitosis as an autonomous structure, or the plasmid is incorporated within the host's genome.

The phrase "heterologous protein" or "recombinantly produced heterologous protein" refers to a peptide or protein of interest produced using cells that do not have

20 an endogenous copy of DNA able to express the peptide or protein of interest. The cells produce the peptide or protein because they have been genetically altered by the introduction of the appropriate nucleic acid sequences. The recombinant peptide or protein will not be found in

25 association with peptides or proteins and other subcellular components normally associated with the cells producing the peptide or protein.

The following terms are used to describe the sequence relationships between two or more nucleic acid

30 molecules or polynucleotides, or between two or more amino acid sequences of peptides or proteins: "reference sequence", "comparison window", "sequence identity", "sequence homology", "percentage of sequence identity", "percentage of sequence homology", "substantial

identity", and "substantial homology". A "reference
sequence" is a defined sequence used as a basis for a
sequence comparison; a reference sequence may be a subset
of a larger sequence, for example, as a segment of a
5   full-length cDNA or gene sequence given in a sequence
listing or may comprise a complete cDNA or gene sequence.

Optimal alignment of sequences for aligning a
comparison window may be conducted, for example, by the
local homology algorithm of Smith and Waterman (1981), by
10 the homology alignment algorithm of Needleman and Wunsch
(1970), by the search for similarity method of Pearson
and Lipman (1988), or by computerized implementations of
these algorithms (GAP, BESTFIT, FASTA, and TFASTA in the
Wisconsin Genetics Software Package Release 7.0, Genetics
15 Computer Group, 575 Science Dr., Madison, Wis.).

As applied to nucleic acid molecules or
polynucleotides, the terms "substantial identity" or
"substantial sequence identity" mean that two nucleic
acid sequences, when optimally aligned (see above), share
20 at least 90 percent sequence identity, preferably at
least 95 percent sequence identity, more preferably at
least 96, 97, 98 or 99 percent sequence identity.

"Percentage nucleotide (or nucleic acid) identity"
or "percentage nucleotide (or nucleic acid) sequence
25 identity" refers to a comparison of the nucleotides of
two nucleic acid molecules which, when optimally aligned,
have approximately the designated percentage of the same
nucleotides. For example, "95% nucleotide identity"
refers to a comparison of the nucleotides of two nucleic
30 acid molecules which when optimally aligned have 95%
nucleotide identity. Preferably, nucleotide positions
which are not identical differ by redundant nucleotide
substitutions (the nucleotide substitution does not
change the amino acid encoded by the particular codon).

As further applied to nucleic acid molecules or polynucleotides, the terms "substantial homology" or "substantial sequence homology" mean that two nucleic acid sequences, when optimally aligned (see above), share

5 at least 90 percent sequence homology, preferably at least 95 percent sequence homology, more preferably at least 96, 97, 98 or 99 percent sequence homology.

"Percentage nucleotide (or nucleic acid) homology" or "percentage nucleotide (or nucleic acid) sequence

10 homology" refers to a comparison of the nucleotides of two nucleic acid molecules which, when optimally aligned, have approximately the designated percentage of the same nucleotides or nucleotides which are not identical but differ by redundant nucleotide substitutions (the

15 nucleotide substitution does not change the amino acid encoded by the particular codon). For example, "95% nucleotide homology" refers to a comparison of the nucleotides of two nucleic acid molecules which when optimally aligned have 95% nucleotide homology.

20   As applied to polypeptides, the terms "substantial identity" or "substantial sequence identity" mean that two peptide sequences, when optimally aligned, such as by the programs GAP or BESTFIT using default gap, share at least 90 percent sequence identity, preferably at least

25 95 percent sequence identity, more preferably at least 96, 97, 98 or 99 percent sequence identity.

"Percentage amino acid identity" or "percentage amino acid sequence identity" refers to a comparison of the amino acids of two polypeptides which, when optimally

30 aligned, have approximately the designated percentage of the same amino acids. For example, "95% amino acid identity" refers to a comparison of the amino acids of two polypeptides which when optimally aligned have 95% amino acid identity. Preferably, residue positions which

are not identical differ by conservative amino acid substitutions. For example, the substitution of amino acids having similar chemical properties such as charge or polarity are not likely to affect the properties of a
5 protein. Examples include glutamine for asparagine or glutamic acid for aspartic acid.

As further applied to polypeptides, the terms "substantial homology" or "substantial sequence homology" mean that two peptide sequences, when optimally aligned,
10 such as by the programs GAP or BESTFIT using default gap, share at least 90 percent sequence homology, preferably at least 95 percent sequence homology, more preferably at least 96, 97, 98 or 99 percent sequence homology.

"Percentage amino acid homology" or "percentage
15 amino acid sequence homology" refers to a comparison of the amino acids of two polypeptides which, when optimally aligned, have approximately the designated percentage of the same amino acids or conservatively substituted amino acids. For example, "95% amino acid homology" refers to
20 a comparison of the amino acids of two polypeptides which when optimally aligned have 95% amino acid homology. As used herein, homology refers to identical amino acids or residue positions which are not identical but differ only by conservative amino acid substitutions. For example,
25 the substitution of amino acids having similar chemical properties such as charge or polarity are not likely to affect the properties of a protein. Examples include glutamine for asparagine or glutamic acid for aspartic acid.
30 The phrase "substantially purified" or "isolated" when referring to a protein (or peptide), means a chemical composition which is essentially free of other cellular components. It is preferably in a homogeneous state although it can be in either a dry or aqueous

solution. Purity and homogeneity are typically determined using analytical chemistry techniques such as polyacrylamide gel electrophoresis or high performance liquid chromatography. A protein (or peptide) which is
5 the predominant species present in a preparation is substantially purified. Generally, a substantially purified or isolated protein (or peptide) will comprise more than 80% of all macromolecular species present in the preparation. Preferably, the protein (or peptide) is
10 purified to represent greater than 90% of all macromolecular species present. More preferably the protein (or peptide) is purified to greater than 95%, and most preferably the protein (or peptide) is purified to essential homogeneity, wherein other macromolecular
15 species are not detected by conventional techniques. A "substantially purified" or "isolated" protein (or peptide) can be separated from an organism, synthetically or chemically produced, or recombinantly produced.

"Biological sample" or "sample" as used herein
20 refers to any sample obtained from a living organism or from an organism that has died. Examples of biological samples include body fluids and tissue specimens.

High stringent hybridization conditions are selected at about 5°C lower than the thermal melting point (Tm)
25 for the specific sequence at a defined ionic strength and pH. The Tm is the temperature (under defined ionic strength and pH) at which 50% of the target sequence hybridizes to a perfectly matched probe. Typically, stringent conditions will be those in which the salt
30 concentration is at least about 0.02 molar at pH 7 and the temperature is at least about 60°C. As other factors may significantly affect the stringency of hybridization, including, among others, base composition and size of the complementary strands, the presence of organic solvents,

i.e. salt or formamide concentration, and the extent of
base mismatching, the combination of parameters is more
important than the absolute measure of any one. High
stringency may be attained, for example, by overnight
5 hybridization at about 68°C in a 6X SSC solution, washing
at room temperature with 6X SSC solution, followed by
washing at about 68°C in a 6X SSC solution then in a 0.6X
SSX solution.

Hybridization with moderate stringency may be
10 attained, for example, by: 1) filter pre-hybridizing and
hybridizing with a solution of 3X sodium chloride, sodium
citrate (SSC), 50% formamide, 0.1M Tris buffer at pH 7.5,
5X Denhardt's solution; 2) pre-hybridization at 37°C for
4 hours; 3) hybridization at 37°C with amount of labeled
15 probe equal to 3,000,000 cpm total for 16 hours; 4) wash
in 2X SSC and 0.1% SDS solution; 5) wash 4X for 1 minute
each at room temperature and 4X at 60°C for 30 minutes
each; and 6) dry and expose to film.

The phrase "selectively hybridizing to" refers to a
20 nucleic acid molecule that hybridizes, duplexes or binds
only to a particular target DNA or RNA sequence when the
target sequences are present in a preparation of total
cellular DNA or RNA. By selectively hybridizing it is
meant that a nucleic acid molecule binds to a given
25 target in a manner that is detectable in a different
manner from non-target sequence under moderate, or more
preferably under high, stringency conditions of
hybridization. "Complementary" or "target" nucleic acid
sequences refer to those nucleic acid sequences which
30 selectively hybridize to a nucleic acid molecule. Proper
annealing conditions depend, for example, upon a nucleic
acid molecule's length, base composition, and the number
of mismatches and their position on the molecule, and
must often be determined empirically. For discussions of

nucleic acid molecule (probe) design and annealing
conditions, see, for example, Sambrook et al. 1989.

It will be readily understood by those skilled in
the art and it is intended here, that when reference is
5   made to particular sequence listings, such reference
includes sequences which substantially correspond to its
complementary sequence and those described including
allowances for minor sequencing errors, single base
changes, deletions, substitutions and the like, such that
10 any such sequence variation corresponds to the nucleic
acid sequence of the signal peptide or other
peptide/protein to which the relevant sequence listing
relates.

The DNA molecules of the subject invention also
15 include DNA molecules coding for protein analogs,
fragments or derivatives of the protein which differ from
naturally-occurring forms (the naturally-occurring
protein) in terms of the identity or location of one or
more amino acid residues (deletion analogs containing
20 less than all of the residues specified for the protein,
substitution analogs wherein one or more residues
specified are replaced by other residues, and addition
analogs wherein one or more amino acid residues is added
to a terminal or medial portion of the protein) and which
25 share the signal property of the naturally-occurring
form. These molecules include: the incorporation of
codons "preferred" for expression by selected
non-mammalian hosts; the provision of sites for cleavage
by restriction endonuclease enzymes; and the provision of
30 additional initial, terminal or intermediate DNA
sequences that facilitate construction of readily
expressed vectors.

As used herein, a "peptide" refers to an amino acid
sequence of three to one hundred amino acids, and

therefore an isolated peptide that comprises an amino acid sequence is not intended to cover amino acid sequences of greater than 100 amino acids. Preferably, the peptides that can be identified and used in

5   accordance with the subject invention (whether they be mimotope or anti-mimotope peptides) are less than 50 amino acids in length, and more preferably the peptides are five to 20 amino acids in length or 20-40 amino acids in length.

10   The peptides can contain any naturally-occurring or non-naturally-occurring amino acids, including the D-form of the amino acids, amino acid derivatives and amino acid mimics, so long as the desired function and activity of the peptide is maintained. The choice of including an

15   (L)- or a (D)-amino acid in the peptides depends, in part, on the desired characteristics of the peptide. For example, the incorporation of one or more (D)-amino acids can confer increased stability on the peptide and can allow a peptide to remain active in the body for an

20   extended period of time. The incorporation of one or more (D)-amino acids can also increase or decrease the pharmacological activity of the peptide.

The peptides may also be cyclized, since cyclization may provide the peptides with superior properties over

25   their linear counterparts.

As used herein, the terms "amino acid mimic" and "mimetic" mean an amino acid analog or non-amino acid moiety that has the same or similar functional characteristic of a given amino acid. For instance, an

30   amino acid mimic of a hydrophobic amino acid is one which is non-polar and retains hydrophobicity, generally by way of containing an aliphatic chemical group. By way of further example, an arginine mimic can be an analog of arginine which contains a side chain having a positive

charge at physiological pH, as is characteristic of the guanidinium side chain reactive group of arginine.

In addition, modifications to the peptide backbone and peptide bonds thereof are also encompassed within the scope of amino acid mimic or mimetic. Such modifications can be made to the amino acid, derivative thereof, non-amino acid moiety or the peptide either before or after the amino acid, derivative thereof or non-amino acid moiety is incorporated into the peptide. What is critical is that such modifications mimic the peptide backbone and bonds which make up the same and have substantially the same spacial arrangement and distance as is typical for traditional peptide bonds and backbones. An example of one such modification is the reduction of the carbonyl(s) of the amide peptide backbone to an amine. A number of reagents are available and well known for the reduction of amides to amines such as those disclosed in Wann et al., JOC, 46:257 (1981) and Raucher et al., Tetrahedron. Lett., 21:14061 (1980). An amino acid mimic is, therefor, an organic molecule that retains the similar amino acid pharmacophore groups as is present in the corresponding amino acid and which exhibits substantially the same spatial arrangement between functional groups.

The substitution of amino acids by non-naturally occurring amino acids and amino acid mimics as described above can enhance the overall activity or properties of an individual peptide based on the modifications to the backbone or side chain functionalities. For example, these types of alterations to the specifically described amino acid substituents and exemplified peptides can enhance the peptide's stability to enzymatic breakdown and increase biological activity. Modifications to the

peptide backbone similarly can add stability and enhance activity.

One skilled in the art, using the above sequences or formulae, can easily synthesize the peptides. Standard procedures for preparing synthetic peptides are well known in the art. The novel peptides can be synthesized using: the solid phase peptide synthesis (SPPS) method of Merrifield (J. Am. Chem. Soc., 85:2149 (1964)) or modifications of SPPS; or, the peptides can be synthesized using standard solution methods well known in the art (see, for example, Bodanzsky, M., Principles of Peptide Synthesis, 2nd revised ed., Springer-Verlag (1988 and 1993)). Alternatively, simultaneous multiple peptide synthesis (SMPS) techniques well known in the art can be used. Peptides prepared by the method of Merrifield can be synthesized using an automated peptide synthesizer such as the Applied Biosystems 431A-01 Peptide Synthesizer (Mountain View, Calif.) or using the manual peptide synthesis technique described by Houghten, Proc. Natl. Acad. Sci., USA 82:5131 (1985).

With these definitions in mind, the subject invention provides an isolated nucleic acid molecule encoding a pancreatic T-type calcium channel. The nucleic acid molecule can be deoxyribonucleic acid (DNA) or ribonucleic acid (RNA, including messenger RNA or mRNA), genomic or recombinant, biologically isolated or synthetic.

The DNA molecule can be a cDNA molecule, which is a DNA copy of a messenger RNA (mRNA) encoding the channel.

An example of such a pancreatic T-type calcium channel is the rat pancreatic T-type calcium channel encoded by the nucleotide sequence as shown in SEQ ID NO:1. The amino acid sequence encoded by this nucleotide sequence is shown in SEQ ID NO:2.

The invention also provides an antisense nucleic acid molecule that is complementary to at least a portion of the mRNA encoding the pancreatic T-type calcium channel. Antisense nucleic acid molecules can be RNA or single-stranded DNA, and can be complementary to the entire mRNA molecule encoding the channel (i.e. of the same nucleotide length as the entire molecule). It may be desirable, however, to work with a shorter molecule. In this instance, the antisense molecule can be complementary to a portion of the entire mRNA molecule encoding the channel. These shorter antisense molecules are capable of hybridizing to the mRNA encoding the entire molecule, and preferably consist of about twenty to about one hundred nucleotides. These antisense molecules can be used to reduce levels of pancreatic T-type calcium channel, by introducing into cells an RNA or single-stranded DNA molecule that is complementary to at least a portion of the mRNA of the channel (i.e. by introducing an antisense molecule). The antisense molecule can base-pair with the mRNA of the channel, preventing translation of the mRNA into protein. Thus, an antisense molecule to the channel can prevent translation of mRNA encoding the channel into a functional channel protein. It may be desirable to place the antisense molecule downstream and under the control or the insulin promoter, so that the antisense will prevent translation of mRNA encoding the T type calcium channel only in islet cells of the pancreas (not affecting brain or heart T type calcium channels). It should also be apparent that 100% prevention of T type calcium channel is not desirable, since a minimal basal Ca^{2+} level is required to be maintained by the T type calcium channel.

More particularly, an antisense molecule
complementary to at least a portion of mRNA encoding a
pancreatic T-type calcium channel can be used to decrease
expression of a functional channel. A cell with a first
5 level of expression of a functional pancreatic T-type
calcium channel is selected, and then the antisense
molecule is introduced into the cell. The antisense
molecule blocks expression of functional pancreatic T-
type calcium channel, resulting in a second level of
10 expression of a functional pancreatic T-type calcium
channel in the cell. The second level is less than the
initial first level.

Antisense molecules can be introduced into cells by
any suitable means. In one embodiment, the antisense RNA
15 molecule is injected directly into the cellular
cytoplasm, where the RNA interferes with translation. A
vector may also be used for introduction of the antisense
molecule into a cell. Such vectors include various
plasmid and viral vectors. For a general discussion of
20 antisense molecules and their use, see Han et al. 1991
and Rossi 1995.

The invention further provides a special category of
antisense RNA molecules, known as ribozymes, having
recognition sequences complementary to specific regions
25 of the mRNA encoding the pancreatic T-type calcium
channel. Ribozymes not only complex with target
sequences via complementary antisense sequences but also
catalyze the hydrolysis, or cleavage, of the template
mRNA molecule. Examples, which are not intended to be
30 limiting, of suitable regions of the mRNA template to be
targeted by ribozymes are any of the homologous regions
identified by comparing the various T-type calcium
channels, and particularly pancreatic β-cell T-type
channels.

Expression of a ribozyme in a cell can inhibit gene expression (such as the expression of a pancreatic T-type calcium channel). More particularly, a ribozyme having a recognition sequence complementary to a region of a mRNA

5   encoding a pancreatic T-type calcium channel can be used to decrease expression of pancreatic T-type calcium channel. A cell with a first level of expression of pancreatic T-type calcium channel is selected, and then the ribozyme is introduced into the cell. The ribozyme

10 in the cell decreases expression of pancreatic T-type calcium channel in the cell, because mRNA encoding the pancreatic T-type calcium channel is cleaved and cannot be translated.

Ribozymes can be introduced into cells by any

15 suitable means. In one embodiment, the ribozyme is injected directly into the cellular cytoplasm, where the ribozyme cleaves the mRNA and thereby interferes with translation. A vector may be used for introduction of the ribozyme into a cell. Such vectors include various

20 plasmid and viral vectors (note that the DNA encoding the ribozyme does not need to be "incorporated" into the genome of the host cell; it could be expressed in a host cell infected by a viral vector, with the vector expressing the ribozyme, for instance). For a general

25 discussion of ribozymes and their use, see Sarver et al. 1990, Chrisey et al. 1991, Rossi et al. 1992, and Christoffersen et al. 1995.

The nucleic acid molecules of the subject invention can be expressed in suitable host cells using

30 conventional techniques. Any suitable host and/or vector system can be used to express the pancreatic T-type calcium channel. For in vitro expression, Xenopus oocytes are preferred. For in vivo expression, the most suitable host cell is a pancreatic β-cell.

Techniques for introducing the nucleic acid
molecules into the host cells may involve the use of
expression vectors which comprise the nucleic acid
molecules. These expression vectors (such as plasmids

5 and viruses; viruses including bacteriophage) can then be
used to introduce the nucleic acid molecules into
suitable host cells. For example, DNA encoding the
pancreatic T-type calcium channel can be injected into
the nucleus of a host cell or transformed into the host

10 cell using a suitable vector, or mRNA encoding the
pancreatic T-type calcium channel can be injected
directly into the host cell, in order to obtain
expression of pancreatic T-type calcium channel in the
host cell.

15 Various methods are known in the art for introducing
nucleic acid molecules into host cells. One method is
microinjection, in which DNA is injected directly into
the nucleus of cells through fine glass needles (or RNA
is injected directly into the cytoplasm of cells).

20 Alternatively, DNA can be incubated with an inert
carbohydrate polymer (dextran) to which a positively
charged chemical group (DEAE, for diethylaminoethyl) has
been coupled. The DNA sticks to the DEAE-dextran via its
negatively charged phosphate groups. These large DNA-

25 containing particles stick in turn to the surfaces of
cells, which are thought to take them in by a process
known as endocytosis. Some of the DNA evades destruction
in the cytoplasm of the cell and escapes to the nucleus,
where it can be transcribed into RNA like any other gene

30 in the cell. In another method, cells efficiently take
in DNA in the form of a precipitate with calcium
phosphate. In electroporation, cells are placed in a
solution containing DNA and subjected to a brief
electrical pulse that causes holes to open transiently in

their membranes. DNA enters through the holes directly into the cytoplasm, bypassing the endocytotic vesicles through which they pass in the DEAE-dextran and calcium phosphate procedures. DNA can also be incorporated into

5 artificial lipid vesicles, liposomes, which fuse with the cell membrane, delivering their contents directly into the cytoplasm. In an even more direct approach, DNA is absorbed to the surface of tungsten microprojectiles and fired into cells with a device resembling a shotgun.

10   Several of these methods, microinjection, electroporation, and liposome fusion, have been adapted to introduce proteins into cells. For review, see Mannino and Gould-Fogerite 1988, Shigekawa and Dower 1988, Capecchi 1980, and Klein et al. 1987.

15   Further methods for introducing nucleic acid molecules into cells involve the use of viral vectors. One such virus widely used for protein production is an insect virus, baculovirus. For a review of baculovirus vectors, see Miller (1989). Various viral vectors have

20 also been used to transform mammalian cells, such as bacteriophage, vaccinia virus, adenovirus, and retrovirus.

       As indicated, some of these methods of transforming a cell require the use of an intermediate plasmid vector.

25 U.S. Patent No. 4,237,224 to Cohen and Boyer describes the production of expression systems in the form of recombinant plasmids using restriction enzyme cleavage and ligation with DNA ligase. These recombinant plasmids are then introduced by means of transformation and

30 replicated in unicellular cultures including procaryotic organisms and eucaryotic cells grown in tissue culture. The DNA sequences are cloned into the plasmid vector using standard cloning procedures known in the art, as described by Sambrook et al. (1989).

Host cells into which the nucleic acid encoding the pancreatic T-type calcium channel has been introduced can be used to produce (i.e. to functionally express) the pancreatic T-type calcium channel. The function of the

5 encoded pancreatic T-type calcium channel can be assayed according to methods known in the art (Wang et al. 1996).

Having identified the nucleic acid molecules encoding pancreatic T-type calcium channels and methods for expressing the pancreatic T-type calcium channels

10 encoded thereby, the invention further provides a method of screening a substance (for example, a compound or inhibitor) for the ability of the substance to modify T-type calcium channel function. The method comprises introducing a nucleic acid molecule encoding the

15 pancreatic T-type calcium channel into a host cell, and expressing the pancreatic T-type calcium channel encoded by the molecule in the host cell. The cell is then exposed to a substance and evaluated to determine if the substance modifies the function of the T-type calcium

20 channel. From this evaluation, substances effective in altering the function of the T-type calcium channel can be found. Such agents may be, for example, calcium channel inhibitors, agonists, or antagonists (for example, mibefradil and mibefradil analogues, amiloride,

25 $NiCl_2$, antisense molecules, and second messengers).

The evaluation of the cell to determine if the substance modifies the function of the T-type calcium channel can be by any means known in the art. The evaluation can comprise the direct monitoring of

30 expression of T-type calcium channel in the host cell, or the evaluation can be indirect and comprise the monitoring of calcium transport by the channel (such as by the methods disclosed by Wang et al. 1996).

The nucleic acid molecules of the subject invention can be used either as probes or for the design of primers to obtain DNA encoding other pancreatic T-type calcium channels by either cloning and colony/plaque

5 hybridization or amplification using the polymerase chain reaction (PCR).

Specific probes derived from SEQ ID NO:1 can be employed to identify colonies or plaques containing cloned DNA encoding a member of the pancreatic T-type

10 calcium channel family using known methods (see Sambrook et al. 1989). One skilled in the art will recognize that by employing such probes under high stringency conditions (for example, hybridization at 42°C with 5X SSPC and 50% formamide, washing at 50-65°C with 0.5X SSPC), sequences

15 having regions which are greater than 90% homologous or identical to the probe can be obtained. Sequences with lower percent homology or identity to the probe, which also encode pancreatic T-type calcium channels, can be obtained by lowering the stringency of hybridization and

20 washing (e.g., by reducing the hybridization and wash temperatures or reducing the amount of formamide employed).

More particularly, in one embodiment, the method comprises selection of a DNA molecule encoding a

25 pancreatic T-type calcium channel, or a fragment thereof, the DNA molecule having a nucleotide sequence as shown in SEQ ID NO:1, and designing an oligonucleotide probe for pancreatic T-type calcium channel based on SEQ ID NO:1. A genomic or cDNA library of an organism is then probed

30 with the oligonucleotide probe, and clones are obtained from the library that are recognized by the oligonucleotide probe so as to obtain DNA encoding another pancreatic T-type calcium channel.

Specific primers derived from SEQ ID NO:1 can be used in PCR to amplify a DNA sequence encoding a member of the pancreatic T-type calcium channel family using known methods (see Innis et al. 1990). One skilled in

5   the art will recognize that by employing such primers under high stringency conditions (for example, annealing at 50-60°C, depending on the length and specific nucleotide content of the primers employed), sequences having regions greater than 75% homologous or identical

10 to the primers will be amplified.

More particularly, in a further embodiment the method comprises selection of a DNA molecule encoding pancreatic T-type calcium channel, or a fragment thereof, the DNA molecule having a nucleotide sequence as shown in

15 SEQ ID NO:1, designing degenerate oligonucleotide primers based on regions of SEQ ID NO:1, and employing such primers in the polymerase chain reaction using as a template a DNA sample to be screened for the presence of pancreatic T-type calcium channel-encoding sequences.

20 The resulting PCR products can be isolated and sequenced to identify DNA fragments that encode polypeptide sequences corresponding to the targeted region of pancreatic T-type calcium channel.

Various modifications of the nucleic acid and amino

25 acid sequences disclosed herein are covered by the subject invention. These varied sequences still encode a functional pancreatic T-type calcium channel. The invention thus further provides an isolated nucleic acid molecule encoding a pancreatic T-type calcium channel,

30 the nucleic acid molecule encoding a first amino acid sequence having at least 90% amino acid identity to a second amino acid sequence, the second amino acid sequence as shown in SEQ ID NO:2. In further embodiments, the first amino acid sequence has at least

95%, 96%, 97%, 98%, or 99% amino acid identity to SEQ ID NO:2.

The invention further provides an isolated DNA oligomer capable of hybridizing to the nucleic acid

5 molecule encoding pancreatic T-type calcium channel according to the subject invention. Such oligomers can be used as probes in a method of detecting the presence of pancreatic T-type calcium channel in a sample. More particularly, a sample can be contacted with the DNA

10 oligomer and the DNA oligomer will hybridize to any pancreatic T-type calcium channel present in the sample, forming a complex therewith. The complex can then be detected, thereby detecting presence of pancreatic T-type calcium channel in the sample.

15 The complex can be detected using methods known in the art. Preferably, the DNA oligomer is labeled with a detectable marker so that detection of the marker after the DNA oligomer hybridizes to any pancreatic T-type calcium channel in the sample (wherein non-hybridized DNA

20 oligomer has been washed away) is detection of the complex. Detection of the complex indicates the presence of pancreatic T-type calcium channel in the sample. As will be readily apparent to those skilled in the art, such a method could also be used quantitatively to assess

25 the amount of pancreatic T-type calcium channel in a sample.

For detection, the oligomers can be labeled with, for example, a radioactive isotope, biotin, an element opaque to X-rays, or a paramagnetic ion. Radioactive

30 isotopes are commonly used and are well known to those skilled in the art. Representative examples include indium-111, technetium-99m, and iodine-123. Biotin is a standard label which would allow detection of the biotin labeled oligomer with avidin. Paramagnetic ions are also

commonly used and include, for example, chelated metal ions of chromium (III), manganese (II), and iron (III). When using such labels, the labeled DNA oligomer can be imaged using methods known to those skilled in the art.

5   Such imaging methods include, but are not limited to, X-ray, CAT scan, PET scan, NMRI, and fluoroscopy. Other suitable labels include enzymatic labels (horseradish peroxidase, alkaline phosphatase, etc.) and fluorescent labels (such as FITC or rhodamine, etc.).

10   The invention further provides an isolated pancreatic T-type calcium channel protein. The protein is preferably encoded by a nucleotide sequence as shown in SEQ ID NO:1. The protein preferably has an amino acid sequence as shown in SEQ ID NO:2. Further provided is an

15 isolated pancreatic T-type calcium channel protein encoded by a first amino acid sequence having at least 90% amino acid identity to a second amino acid sequence, the second amino acid sequence as shown in SEQ ID NO:2. In further embodiments, the first amino acid sequence has

20 at least 95%, 96%, 97%, 98%, or 99% amino acid identity to SEQ ID NO:2.

The pancreatic T-type calcium channel molecule of the subject invention can include a leader sequence for targeting of the pancreatic T-type calcium channel

25 protein to the desired part of a cell.

It should be readily apparent to those skilled in the art that a met residue may need to be added to the amino terminal of the amino acid sequence of the mature pancreatic T-type calcium channel protein (i.e., added to

30 SEQ ID NO:2) or an ATG added to the 5' end of the nucleotide sequence (i.e., added to SEQ ID NO:1), in order to express the channel in a host cell. The met version of the mature channel is thus specifically

intended to be covered by reference to SEQ ID NO:1 or SEQ ID NO:2.

The invention further provides an antibody or fragment thereof specific for the pancreatic T-type calcium channel of the subject invention. Antibodies of the subject invention include polyclonal antibodies and monoclonal antibodies capable of binding to the pancreatic T-type calcium channel, as well as fragments of these antibodies, and humanized forms. Humanized forms of the antibodies of the subject invention may be generated using one of the procedures known in the art such as chimerization. Fragments of the antibodies of the present invention include, but are not limited to, the Fab, the F(ab')$_2$, and the Fc fragments.

The invention also provides hybridomas which are capable of producing the above-described antibodies. A hybridoma is an immortalized cell line which is capable of secreting a specific monoclonal antibody.

In general, techniques for preparing polyclonal and monoclonal antibodies as well as hybridomas capable of producing the desired antibody are well known in the art (see Campbell 1984 and St. Groth et al. 1980). Any animal (mouse, rabbit, etc.) which is known to produce antibodies can be immunized with the antigenic pancreatic T-type calcium channel (or an antigenic fragment thereof). Methods for immunization are well known in the art. Such methods include subcutaneous or intraperitoneal injection of the protein. One skilled in the art will recognize that the amount of the protein used for immunization will vary based on the animal which is immunized, the antigenicity of the protein, and the site of injection.

The protein which is used as an immunogen may be modified or administered in an adjuvant in order to

increase the protein's antigenicity. Methods of
increasing the antigenicity of a protein are well known
in the art and include, but are not limited to, coupling
the antigen with a heterologous protein (such as a
5 globulin or beta-galactosidase) or through the inclusion
of an adjuvant during immunization.

For monoclonal antibodies, spleen cells from the
immunized animals are removed, fused with myeloma cells,
such as SP2/O-Ag 15 myeloma cells, and allowed to become
10 monoclonal antibody producing hybridoma cells.

Any one of a number of methods well known in the art
can be used to identify the hybridoma cell which produces
an antibody with the desired characteristics. These
include screening the hybridomas with an ELISA assay,
15 western blot analysis, or radioimmunoassay (Lutz et al.
1988).

Hybridomas secreting the desired antibodies are
cloned and the class and subclass are determined using
procedures known in the art (Campbell 1984).

20 For polyclonal antibodies, antibody containing
antisera is isolated from the immunized animal and is
screened for the presence of antibodies with the desired
specificity using one of the above-described procedures.

The present invention further provides the above-
25 described antibodies in detectably labeled form.
Antibodies can be detectably labeled through the use of
radioisotopes, affinity labels (such as biotin, avidin,
etc.), enzymatic labels (such as horseradish peroxidase,
alkaline phosphatase, etc.), fluorescent labels (such as
30 FITC or rhodamine, etc.), paramagnetic atoms, etc.
Procedures for accomplishing such labeling are well known
in the art, for example see Sternberger et al. 1970,
Bayer et al. 1979, Engval et al. 1972, and Goding 1976.

The labeled antibodies or fragments thereof of the present invention can be used for in vitro, in vivo, and in situ assays to identify cells or tissues which express pancreatic T-type calcium channel, to identify samples containing pancreatic T-type calcium channel, or to detect the presence of pancreatic T-type calcium channel in a sample. More particularly, the antibodies or fragments thereof can thus be used to detect the presence of pancreatic T-type calcium channel in a sample, by contacting the sample with the antibody or fragment thereof. The antibody or fragment thereof binds to any pancreatic T-type calcium channel present in the sample, forming a complex therewith. The complex can then be detected, thereby detecting the presence of pancreatic T-type calcium channel in the sample. As will be readily apparent to those skilled in the art, such a method could also be used quantitatively to assess the amount of pancreatic T-type calcium channel in a sample. As should also be readily apparent, such an antibody can also be used to decrease levels of functional T type calcium channels, by blocking the channel. Such antibodies can therefore be used in the methods of the subject invention to modify levels of functional T type calcium channels in pancreatic beta cells.

Further provided is a composition comprising the pancreatic T-type calcium channel protein and a compatible carrier.

In the methods of the invention, tissues or cells are contacted with or exposed to the composition of the subject invention or a compound. In the context of this invention, to "contact" tissues or cells with or to "expose" tissues or cells to a composition or compound means to add the composition or compound, usually in a liquid carrier, to a cell suspension or tissue sample,

either in vitro or ex vivo, or to administer the composition or compound to cells or tissues within an animal (including humans).

For therapeutics, methods of modifying insulin
5 secretion by pancreatic beta cells, methods of treating type II diabetes, methods of modifying basal calcium levels in cells, methods of modifying the action potential of L type calcium channels in cells, methods of modifying pancreatic beta cell death, methods of
10 modifying pancreatic beta cell proliferation, and methods of modifying calcium influx through L type calcium channels in cells, each of the methods comprising modifying levels of functional T type calcium channels in the cells, are provided. The formulation of therapeutic
15 compositions and their subsequent administration is believed to be within the skill in the art. In general, for therapeutics, a patient suspected of needing such therapy is given a composition in accordance with the invention, commonly in a pharmaceutically acceptable
20 carrier, in amounts and for periods which will vary depending upon the nature of the particular disease, its severity and the patient's overall condition. The pharmaceutical compositions of the present invention may be administered in a number of ways depending upon
25 whether local or systemic treatment is desired and upon the area to be treated. Administration may be topical (including ophthalmic, vaginal, rectal, intranasal, transdermal), oral or parenteral. Parenteral administration includes intravenous drip or infusion,
30 subcutaneous, intraperitoneal or intramuscular injection, pulmonary administration, e.g., by inhalation or insufflation, or intrathecal or intraventricular administration.

Formulations for topical administration may include transdermal patches, ointments, lotions, creams, gels, drops, suppositories, sprays, liquids and powders. Conventional pharmaceutical carriers, aqueous, powder or

5 oily bases, thickeners and the like may be necessary or desirable. Coated condoms, gloves and the like may also be useful.

Compositions for oral administration include powders or granules, suspensions or solutions in water or non-

10 aqueous media, capsules, sachets or tablets. Thickeners, flavoring agents, diluents, emulsifiers, dispersing aids or binders may be desirable.

Compositions for parenteral, intrathecal or intraventricular administration may include sterile

15 aqueous solutions which may also contain buffers, diluents and other suitable additives.

In addition to such pharmaceutical carriers, cationic lipids may be included in the formulation to facilitate uptake. One such composition shown to

20 facilitate uptake is LIPOFECTIN (BRL, Bethesda MD).

Dosing is dependent on severity and responsiveness of the condition to be treated, with course of treatment lasting from several days to several months or until a cure is effected or a diminution of disease state is

25 achieved. Optimal dosing schedules can be calculated from measurements of drug accumulation in the body. Persons of ordinary skill can easily determine optimum dosages, dosing methodologies and repetition rates. Optimum dosages may vary depending on the relative

30 potency of individual compositions, and can generally be calculated based on IC_{50}'s or EC_{50}'s in in vitro and in vivo animal studies. For example, given the molecular weight of compound (derived from oligonucleotide sequence and/or chemical structure) and an effective dose such as

an IC_{50}, for example (derived experimentally), a dose in mg/kg is routinely calculated.

The methods of the subject invention are based on the discovery that regulation of T type calcium channels directly modifies basal calcium levels in cells, which in turn regulates L type calcium channel activity, which in turn regulates insulin secretion and cell death, which in turn treats type II diabetes. The methods of the subject invention are further based on the discovery that regulation of T type calcium channels directly affects basal and glucose-induced insulin secretion.

T type calcium channels belong to the family of low voltage activated calcium channels. Modifying (increasing or decreasing) "levels" of functional T type calcium channels refers to modifying expression of the T type calcium channel gene, modifying activity of the T type calcium channel such as by inhibiting the function of the channel, and/or modifying the formation of active membrane-spanning T type calcium channels. As used herein, "functional" refers to the synthesis and any necessary post-translational processing of a calcium channel molecule in a cell so that the channel is inserted properly in the cell membrane and is capable of conducting calcium ions in accordance with a low voltage activated channel.

The invention thus provides a method of modifying insulin secretion by pancreatic beta cells, the method comprising modifying levels of T type calcium channels in the pancreatic beta cells.

Levels of T type calcium channels in the pancreatic beta cells can be modified by various methods, at the gene and protein and "functional calcium channel" levels. In one embodiment, the levels are modified by modifying T type calcium channel gene expression of the T type

calcium channel in the cells. This can be accomplished by exposing the cells to a compound which modifies T type calcium channel gene expression of the calcium channel. The compound could be, for example, an antisense

5 oligonucleotide targeted to the T type calcium channel gene. In a similar embodiment, the compound which modifies T type calcium channel gene expression of the T type calcium channel could be a ribozyme.

Other methods for modifying T type calcium channel

10 gene expression could also involve site-directed mutagenesis of the T type calcium channel gene to prevent expression of the T type calcium channel, or various gene therapy techniques.

Levels, in particular activity, of T type calcium

15 channels in the cell can also be modified by exposing the cells to an inhibitor of the T type calcium channel. Such inhibitors include, for example, mibefradil, mibefradil analogs, amiloride, $NiCl_2$, and second messengers which regulate activity of the T type calcium

20 channels. Other inhibitors of the T type calcium channel could also readily be identified by screening methods (including the method described above). In addition to chemical inhibitors, peptide inhibitors could also be identified with screening methods (for example, using

25 phage display libraries and other peptide screening methods).

"Mibefradil analogs", as used herein are meant to include compounds having the formula:
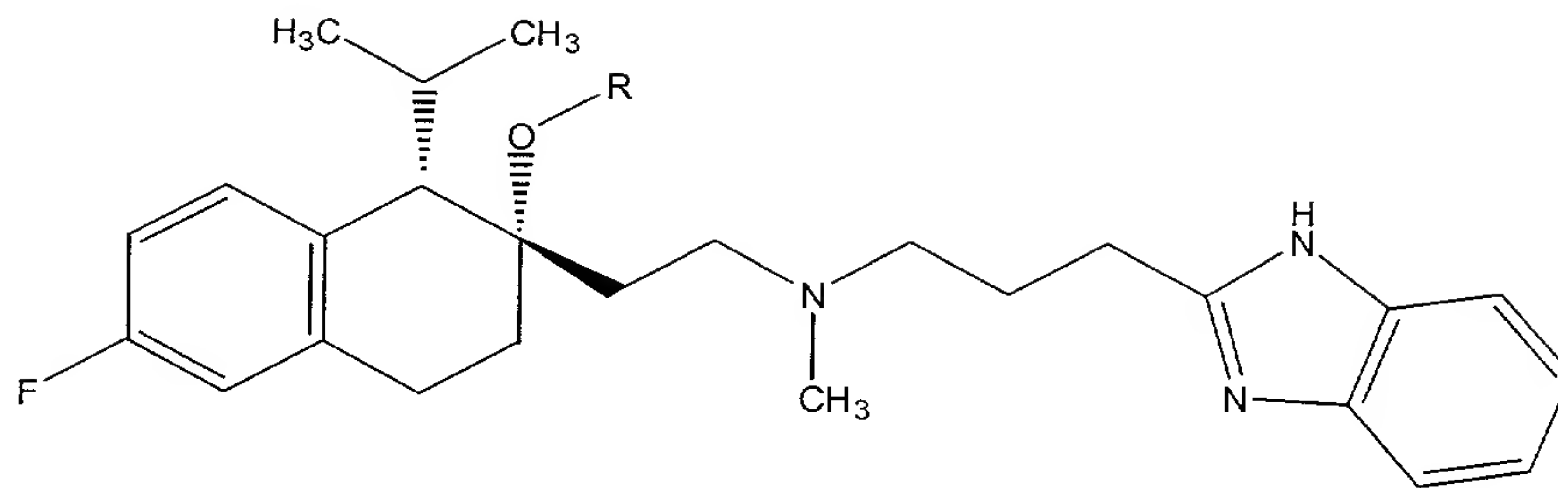
wherein R is hydrogen, alkyl, or a moiety having the
formula C(O)R', where R' is alkyl or aryl. In the above
5 formulae, alkyl is meant to include linear alkyls,
particularly C1-C12 linear alkyls (e.g., methyl, ethyl,
n-propyl, n-pentyl, n-hexyl, n-heptyl, n-octyl, n-nonyl,
and the like), branched alkyls, particularly C1-C12
branched alkyls (e.g., isobutyl, isopentyl, neopentyl,
10 hex-2-yl, hex-3-yl, hept-2-yl, hept-3-yl, and the like),
and cycloalkyls, particularly C1-C8 cycloalkyls (e.g.,
cyclopentyl, cyclohexyl, cycloheptyl, 4-methylcyclohexyl,
and the like). These alkyl groups can be substituted or
unsubstituted. When substituted, suitable substituents
15 include, for example, aryl groups, halogen atoms, hydroxy
groups, alkoxy groups, carboxylic acid groups, amine
groups, and the like, as well as combinations of these
substituents. Mibefradil analogs which are particularly
well suited to blocking (inhibiting) the activity of T-
20 type calcium channels but not blocking the activity of L-
type calcium channels are those having the formula:

25
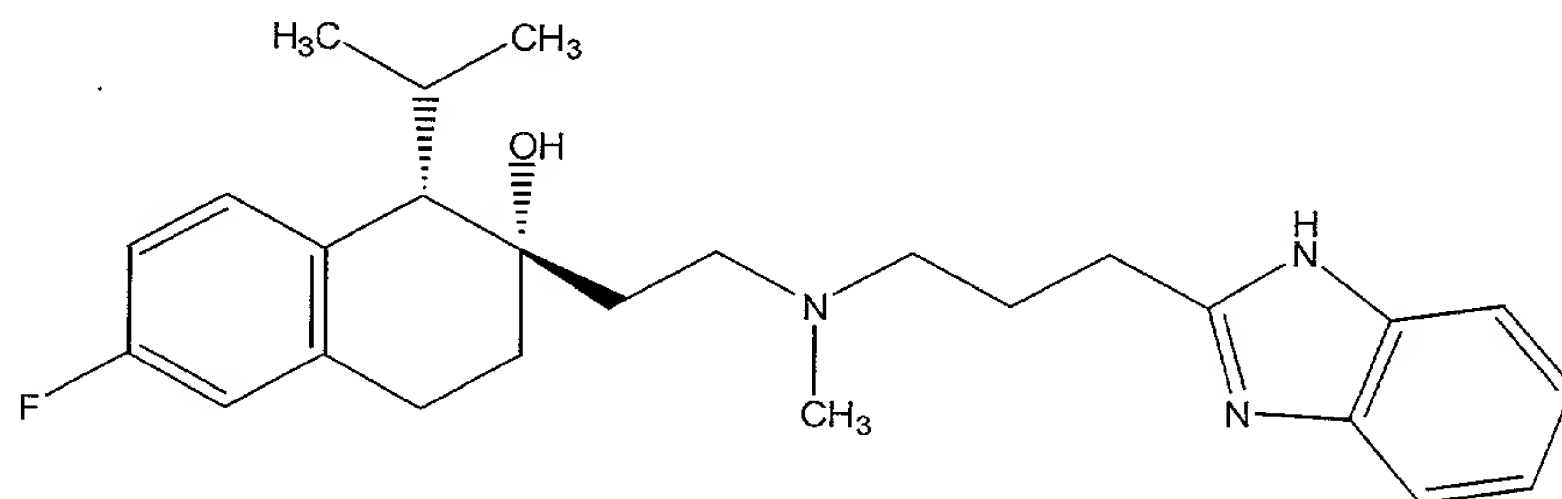
and those having the formula:
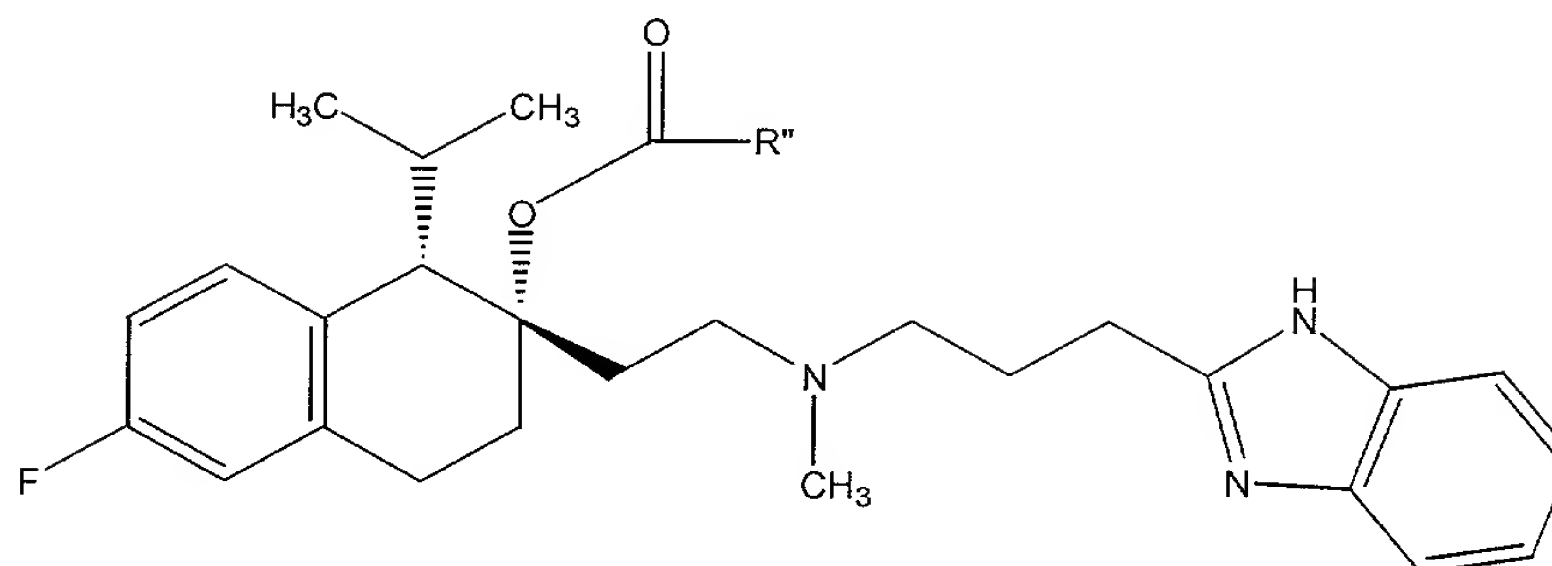
5 in which R" is an unsubstituted alkyl group or a
substituted alkyl group which does not contain an alkoxy
substituent. "Mibefradil analogs" are also meant to
include compounds having the above formulae which are
substituted at other positions in the structure, for
10 example, on the benzimidazole phenyl moiety, at a
benzimidazole nitrogen, at other positions of the
tetrahydronaphthyl ring, etc. Also included within the
meaning of "mibefradil analogs" are compounds having the
above formulae in which the F is replaced with another
15 substituent, such as another halogen. Also included
within the meaning of "mibefradil analogs" are compounds
having the above formulae in which the amine methyl group
or the isopropyl group or both are replaced with other
substituents, such as other alkyl moieties.
20 Additionally, "mibefradil analogs" are meant to include
those compounds which are generically described and/or
specifically disclosed in U.S. Patent No. 4,808,605,
which is hereby incorporated by reference. Further,
"mibefradil analogs" are meant to include
25 pharmaceutically acceptable salts of the derivatives
described above. Illustrative pharmaceutically
acceptable salts are salts formed with hydrochloric acid,
hydrobromic acid, nitric acid, sulphuric acid, phosphoric
acid, citric acid, formic acid, maleic acid, acetic acid,

succinic acid, tartaric acid, methanesulphonic acid, p-toluenesulphonic, and the like.

Mibefradil analogs can be made by following the general procedures described in, for example, U.S. Patent Nos. 4,808,605, 5,910,606, 5,892,055, 5,811,557, 5,811,556, and 5,808,088, each of which is hereby incorporated by reference.

Levels of T type calcium channels in the cell can also be modified by exposing the cells to a compound which interferes with membrane T type calcium channel formation.

Levels of functional T type calcium channel could also be modified by use of molecules which bind to transcription regulators of the T type calcium channel gene (such as the promoter region of the gene).

The invention further provides a method of treating type II diabetes in a subject (human or animal), the method comprising administering to the subject an amount of a compound effective to modify levels of T type calcium channels in the pancreatic beta cells of the subject. As above, the compound may modify levels of T type calcium channels by modifying T type calcium channel gene expression of the calcium channel, or by inhibiting the T type calcium channel, or by interfering with membrane T type calcium channel formation.

In the context of this invention "modulation" or "modifying" means either inhibition or stimulation. This modulation can be measured in ways which are routine in the art, for example by Northern blot assay of mRNA expression, Western blot assay of protein expression, or calcium channel activity assay.

The compounds and/or inhibitors used in the methods of the subject invention encompass any pharmaceutically acceptable salts, esters, or salts of such esters, or any

other compound/inhibitor which, upon administration to an animal including a human, is capable of providing (directly or indirectly) the biologically active metabolite or residue thereof. Accordingly, for example,

5 the disclosure is also drawn to prodrugs and pharmaceutically acceptable salts of the compounds and/or inhibitors used in the subject invention, pharmaceutically acceptable salts of such prodrugs, and other bioequivalents.

10   In regard to prodrugs, the compounds and/or inhibitors for use in the invention may additionally or alternatively be prepared to be delivered in a prodrug form. The term prodrug indicates a therapeutic agent that is prepared in an inactive form that is converted to

15 an active form (i.e., drug) within the body or cells thereof by the action of endogenous enzymes or other chemicals and/or conditions.

    In regard to pharmaceutically acceptable salts, the term pharmaceutically acceptable salts refers to

20 physiologically and pharmaceutically acceptable salts of the compounds and/or inhibitors used in the subject invention: i.e., salts that retain the desired biological activity of the parent compound and do not impart undesired toxicological effects thereto.

25   Drugs, such as peptide drugs, which inhibit the T type calcium channel or which interfere with functional T type calcium channel formation can be identified by other methods also. For example, a monoclonal antibody can be prepared which specifically hybridizes to the T type

30 calcium channel, thereby interfering with activity and/or channel formation. Once a monoclonal antibody which specifically hydridizes to the T type calcium channel is identified, the monoclonal (which is itself a compound or inhibitor which can be used in the subject invention) can

be used to identify peptides capable of mimicking the inhibitory activity of the monoclonal antibody. One such method utilizes the development of epitope libraries and biopanning of bacteriophage libraries. Briefly, attempts

5 to define the binding sites for various monoclonal antibodies have led to the development of epitope libraries. Parmley and Smith developed a bacteriophage expression vector that could display foreign epitopes on its surface (Parmley, S.F. & Smith, G.P., Gene 73:305-318

10 (1988)). This vector could be used to construct large collections of bacteriophage which could include virtually all possible sequences of a short (e.g. six-amino-acid) peptide. They also developed biopanning, which is a method for affinity-purifying phage displaying

15 foreign epitopes using a specific antibody (see Parmley, S.F. & Smith, G.P., Gene 73:305-318 (1988); Cwirla, S.E., et al., Proc Natl Acad Sci USA 87:6378-6382 (1990); Scott, J.K. & Smith, G.P., Science 249:386-390 (1990); Christian, R.B., et al., J Mol Biol 227:711-718 (1992);

20 Smith, G.P. & Scott, J.K., Methods in Enzymology 217:228-257 (1993)).

After the development of epitope libraries, Smith et al. then suggested that it should be possible to use the bacteriophage expression vector and biopanning technique

25 of Parmley and Smith to identify epitopes from all possible sequences of a given length. This led to the idea of identifying peptide ligands for antibodies by biopanning epitope libraries, which could then be used in vaccine design, epitope mapping, the identification of

30 genes, and many other applications (Parmley, S.F. & Smith, G.P., Gene 73:305-318 (1988); Scott, J.K., Trends in Biochem Sci 17:241-245 (1992)).

Using epitope libraries and biopanning, researchers searching for epitope sequences found instead peptide

sequences which mimicked the epitope, i.e., sequences which did not identify a continuous linear native sequence or necessarily occur at all within a natural protein sequence. These mimicking peptides are called

5 mimotopes. In this manner, mimotopes of various binding sites/proteins have been found.

The sequences of these mimotopes, by definition, do not identify a continuous linear native sequence or necessarily occur in any way in a naturally-occurring

10 molecule, i.e. a naturally occurring protein. The sequences of the mimotopes merely form a peptide which functionally mimics a binding site on a naturally-occurring protein.

Many of these mimotopes are short peptides. The

15 availability of short peptides which can be readily synthesized in large amounts and which can mimic naturally-occurring sequences (i.e. binding sites) offers great potential application.

Using this technique, mimotopes to a monoclonal

20 antibody that recognizes T type calcium channels can be identified. The sequences of these mimotopes represent short peptides which can then be used in various ways, for example as peptide drugs that bind to T type calcium channels and decrease the activity of T type calcium

25 channels. Once the sequence of the mimotope is determined, the peptide drugs can be chemically synthesized.

MATERIALS AND METHODS

30   Cell Culture - INS-1 cells were cultured in RPMI 1640 medium containing 10% FBS, 25 U/ml penicillin, 25 mg/ml streptomycin and 50 μM mercaptoethanol in an atmosphere of 5% CO_2 in air, at 37°C for 2-5 days before recording.

Islet cell preparation - Pancreases of Sprague-Dawley rats (Charles River Laboratory, Wilmington, MA) were removed after intrapancreatic perfusion with 2 ml of Hanks' solution (Gibco BRL, Grand

5 Island, NY) containing collagenase (4 mg/ml, Boehringer Mannheim, Indianapolis, IN), DNase I (10 μg/ml, Sigma, St. Louis, MO), $CaCl_2$ (1.28 mM) and bovine serum albumin (1 mg/ml, Gibco BRL). The pancreatic tissue was incubated at 37°C for 20 min and then washed five times with

10 enzyme-free Hanks' solution. Islets were picked up and treated with 0.1% pancreatin (Sigma) for five minutes at 37°C. Single cells were obtained by triturating the islets with plastic pipette tips and then they were transferred into 35 mm culture dishes. Cells were

15 cultured in RPMI 1640 medium (Gibco BRL) containing 5 mM glucose, 10% FBS and P/S at 37°C, 5% CO_2 for 2-5 days before experiments.

Isolation of RNA - Total RNA was isolated from cultured INS-1 cells and from various freshly excised rat

20 tissues by the guanidinium isothiocyanate/phenol procedure (Chomczynsk and Sacchi 1987). Poly-A RNA was isolated from total RNA by two successive passes over an oligo (dT)-cellulose spin column (Ambion, Austin, TX).

Cloning of cDNA Encoding α1 Subunit of T-type Ca^{2+}

25 channel in INS-1 - First strand cDNA was prepared using 2 μg of INS-1 cell mRNA and M-MLV reverse transcriptase (Gibco BRL) with the poly-dT primers. The first 433 bp DNA fragment of the channel was deduced with PCR using the degenerate primers (forward)(SEQ ID NO:6) 5'-

30 TNGC(A/C/T)ATGGAG(C/A)GNCC(C/T)-3' and (backward)(SEQ ID NO:7) 5'-CTT(C/G/T)CCCTTGAA(G/C)A(G/A)CTG)-3' based on conserved voltage-dependent Ca^{2+} channel α_1 subunit sequences in domain III. Using the Marathon™ cDNA Amplification Kit (Clontech, Palo Alto, CA), the 3'- and

5'- rapid amplifications of cDNA end-PCR (RACE-PCR) were performed to obtain the entire gene of the α_1 subunit of the channel. For the 5'-RACE-PCR, the forward primer was an adapter primer, the backward primer was (SEQ ID NO:8)

5   5'-CCGCTGTCGGAGACCATGGAGACC-3'; for the 3'-RACE, the forward primer was (SEQ ID NO:9) 5'-AGCGGCCCAAAATTGACCCCCACAG-3' and the backward primer was poly-dT. The RT-PCR products were subcloned into pT-Adv Vector (Clontech) and dideoxynucleotide sequencing assay

10 was performed with a dsDNA Cycle Sequencing System (Gibco BRL).

Tissue distribution - The gene expression of T-type Ca^{2+} channels deduced from β-cells was examined in rat brain, heart, kidney, and liver using an RT-PCR assay.

15 The primers used for the RT-PCR were (SEQ ID NO:10) 5'-GAAGATGCGAGTGGACAG-3' (forward) and (SEQ ID NO:11) 5'-CTGTGGCGATGGTCACTG-3' (backward). The PCR products were detected by agarose gel electrophoresis on a 1% gel.

Genome walking - The genome walker library

20 (Clontech) was used as a template in nested PCR reactions with gene-specific primers (GSP) and the adapter primers (AP) provided with the kit. The first PCR reaction was carried out in 5 tubes, each having a total volume of 50μl: 5μl 10X PCR reaction buffer, 1 μl dNTP (10 mM

25 each), 2.2μl Mg(OAc)$_2$ (25 mM), 1 μl AP1 (10 μM), 1 μl GSP1, 1 μl Advantage Genomic Polymerase Mix (50X), and 37.8 μl water. The following two-step cycle parameters were used: (Step 1) 7 cycles of denaturing at 94°C for 25 sec., annealing and extension at 72°C for 4 min. (Step 2)

30 32 cycles of denaturing at 94°C for 25 sec., annealing and extension at 67°C for 4 min. After the second step cycle, the samples were held at 67°C for 4 min. The second PCR reaction was carried out under the reaction condition similar to the first PCR reaction except using

AP2, GSP2. In addition, the templates used were 1 μl of 1:50 dilution of each primary PCR reaction. The two step cycles were similar to the first PCR reaction except 5 cycles at the first step and 22 cycles at the second

5 step.

Oocyte electrophysiology - cRNA transcripts were synthesized from BssH II linearized pT-Adv cDNA templates using T7 RNA polymerase (Ambion). Defolliculated Xenopus laevis were injected with 25 ng pT-Adv cRNA. Three to

10 five days after injection, two-electrode voltage-clamp recording was performed using a Warner OC-725C amplifier (Warner Instrument Corp., Hamden, CT). Data were acquired and analyzed with Pulse/PulseFit software (HEKA, Lambrecht/Pfalz, Germany). The bath solution contained

15 the following: 40 mM Ca(OH)$_2$, 50 mM NaOH, 2 mM TEA-Cl, 1mM KOH, 0.1 mM EDTA and 5 mM HEPES, adjusted to pH 7.4 with methanesulphonate. Boltzmann fits were calculated using Prism (GraphPad). Results are presented as mean ± s.d. unless otherwise stated.

20 β-cell Electrophysiological recording - The whole-cell recordings were carried out by the standard "giga-seal" patch clamp technique (Hamill et al.). The whole-cell recording pipettes were made of hemocapillaries (Warner), pulled by a two-stage puller

25 (PC-10, Narishige International, New York, NY), and heat polished with a microforge (MF200-1, World Precision Instruments, Sarasota, FL) before use. Pipette resistance was in the range of 2-5 MΩ in the internal solution. The recordings were performed at room temperature (22-25°C).

30 Currents were recorded using an EPC-9 patch-clamp amplifier (HEKA) and filtered at 2.9 kHz. Data were acquired with Pulse/PulseFit software (HEKA). Voltage-dependent currents were corrected for linear leak

and residual capacitance by using an on-line P/n subtraction paradigm.

Drugs - Mibefradil ((1S,2S)-2-[2-[[3-(2-Benzimidazolyl)propyl]methyl-amino]ethyl]-6-fluoro-1,2,3,4-tetrahydro-1-isopropyl-2-napthyl methoxy-acetate dihydrochloride) was kindly provided by Dr. J.-P. Clozel (Hoffmann LaRoche, Basel, Switzerland), and can be synthesized according to the methods disclosed in U.S. Patent Nos. 5,892,055, 5,811,557, 5,811,556, and 5,808,088. U.S. Patent No. 4,808,605 describes mibefradil compounds suitable for use in the subject invention.

The free alcohol Des-methoxyacetyl mibefradil (1S,2S)-2-[2-[[3-(2-Benzimidazolyl)propyl] methylamino]ethyl]-6-fluoro-1,2,3,4-tetrahydro-1-isopropyl-2-napthyl hydroxy hydrochloride) was prepared by alkaline hydrolysis: 14.2 mg mibefradil hydrochloride was dissolved in 4 ml methanol + 1 ml 10 N aqueous sodium hydroxide mixture (5 mM was the final concentration of mibefradil). The solution was warmed in a boiling water bath for 10 min. The reaction was followed by mass spectrometry. Upon completion of the hydrolysis, as determined from the mass spectra, the solution was neutralized with 5 M aqueous hydrochloric acid. The slight loss of methanol that occurred by evaporation during the reaction was corrected by adding water to keep the total volume of 5 ml.

Solutions - The extracellular solution used in whole-cell Ca^{2+} current recording contained (in mM): 10 $CaCl_2$, 110 tetraethylammonium-Cl (TEA-Cl), 10 CsCl, 10 N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid (HEPES), 40 sucrose, 0.5 3,4-diaminopyridine, pH 7.3. The intracellular solution contained (in mM): 130 N-methyl-D-glucamine, 20 EGTA (free acid), 5 bis (2-aminophenoxy)

ethane-N, N, N', N'-tetraacetate (BAPTA), 10 HEPES, 6

MgCl$_2$, 4 Ca(OH)$_2$, pH was adjusted to 7.4 with

methanesulfonate. 2 mM Mg-ATP was included in the pipette

solution to minimize rundown of L-type Ca^{2+} currents. For

5   Perforated-patch recording, the extracellular solution

contained (in mM): 26 Sucrose, 30 TEA-Cl, 10 HEPES, 5

KCl, 2 CaCl$_2$, MgCl$_2$, pH 7.3. The pipette solution

contained (in mM): 65 CsOH, 65 CsMS, 20 sucrose, 10

HEPES, 10 MgCl$_2$, 1 Ca(OH)$_2$, pH 7.4.

10   Mass Spectrometric Analysis - A VG 70-250 SEQ

instrument (VG Analytical, Manchaster, UK) was used with

fast atom bombardment (FAB) ionization mode to obtain

mass spectra of the mibefradil and dm-mibefradil.

Cultured INS-1 cells were treated with 20 μM mibefradil

15 for various lengths of time under each experimental

condition. The cell pellets were collected after washing

three times with PBS and resuspended in 0.5 ml media for

mass spectrometric analysis. For a 50 μl cell sample, 20

μl internal standard solution (40 μM verapamil, MW:454)

20 and 5 μl glycerol was added, and 4 μl of this mixture was

used for FAB-MS. Several positive ion spectra were

recorded in the mass range m/z 750-100 at a mass

resolution of 1000, and a scan speed of 2 second/decade.

For mibefradil, m/z 496 was the dominant ion (M+H)$^+$

25 accompanied with a less intense sodiated molecular ion

m/z 518. The concentrations of the mibefradil and

hydrolyzed mibefradil were obtained by comparing the

intensities of m/z 496 and 424 were to the intensity of

m/z 455. For calibration, a standard solution of 50 μM

30 drug was subjected to mass spectrometric analysis.

Separation of cytosolic and membrane components -

After washing out mibefradil from the bath solution, the

cells were collected and the membranes were broken down

by vortexing the cells in a solution containing 5% acetic

acid/CH_3CN. The mixture was then spun and the supernatant
collected and defined as non-membrane associated
components. Pellets were re-suspended in 5 x volume of
NaOH (10 N):methanol (1:7) solution at 37°C for 5 min.
5   The mixture was neutralized with 0.5 M HCl and spun down.
The remaining pellet and the supernatant were collected
separately.

Statistics - All data is presented as mean ± s.d.
and the student's t-test was used to calculate p values
10 where given.

EXAMPLE I

Identification and Cloning of a Pancreatic
T-type Calcium Channel

15   The subject invention provides a cDNA encoding a
T-type Ca^{2+} channel α_1 subunit derived from the
rat insulin secreting cell line, INS-1, which has been
identified and sequenced. The sequence of the cDNA
indicates a protein composed of 2288 amino acids (SEQ ID
20 NO:2), sharing 96.3% identity to the neuronal T-type Ca^{2+}
channel α_1 subunit (α_1G). The transmembrane domains of the
protein are highly conserved but the isoform contains
three distinct regions as well as 10 single amino acid
substitutions in other regions. Sequencing rat genomic
25 DNA revealed that this is an alternative splice isoform
of α_1G. Using specific primers and reverse transcription
polymerase chain reaction (RT-PCR) it was demonstrated
that both splice variants are expressed in rat islets.
The isoform deduced from INS-1 was also expressed in
30 brain, neonatal heart and kidney. Functional expression
of this α_1G isoform in Xenopus oocytes generated
low-voltage activated Ca^{2+} currents. These results provide
the molecular biological basis for studies of function of

T-type Ca^{2+} channels in β-cells where these channels play critical roles in diabetes.

The cloning and tissue distribution of an isoform of the T-type Ca^{2+} channel (α_1G-INS) derived from the rat
5   insulin-secreting cell line, INS-1 (Asfari et al. 1992), is described further below.

Based on the conserved amino acid sequence comprising the six transmembrane segments in repeat III of the previously cloned α_1-subunit (Stea et al. 1995),
10 degenerate primers were designed to deduce the cDNA sequence of voltage-dependent Ca^{2+} channel from INS-1 which expresses a high level of T-type Ca^{2+} current (Bhattacharjee et al. 1997). A 433 base pair (bp) DNA fragment was obtained. The rapid amplification of cDNA
15 ends (RACE) strategy was then used to obtain the entire sequence of the channel. The full length cDNA (SEQ ID NO:1) encodes a protein containing 2288 amino acids (SEQ ID NO:2).

The T-type Ca^{2+} channel gene deduced from β-cells
20 shares 96.3% amino acid identity with α_1G, the neuronal isoform of T-type Ca^{2+} channel (Perez-Reyes et al. 1998). The four intramolecular homologous transmembrane domains of β-cell T-type Ca^{2+} channel α_1 subunit are identical (except glycine 1667) to α_1G, with each repeat containing
25 six putative membrane-spanning regions (S1-S6) and a pore-forming region (P-loop). The other highly conserved region is located at the intracellular loop between repeat I and II, where a section of histidine-rich chain is present in the β-cell derived T-type Ca^{2+} channel gene
30 as well as in neuronal and cardiac T-type Ca^{2+} channel genes. This structure in the $loop_{I-II}$ has not been observed in the protein sequences of known high voltage activated Ca^{2+} channels.

In addition to the single amino acids that differ from α_1G, the T-type Ca^{2+} channel gene derived from β-cells contains three unique regions that differ from the amino acid sequence of α_1G. These regions are located
5 at the N-terminal amino acids (aa1-34 of SEQ ID NO:2), intracellular loop I_{II-III} (aa971-994 of SEQ ID NO:2) and intracellular loop L_{III-IV} (aa1570-1588 of SEQ ID NO:2).

Although the amino acid sequence of the deduced channel is entirely different from the α_1G in the
10 N-terminal region (aa1-34 of SEQ ID NO:2), the nucleotide sequences at this region are almost identical except for 4 single nucleotide insertions which are shown in Fig. 1A. These four single nucleotide insertions determine a different start codon as well as those of the amino acid
15 sequences.

To resolve the relationship between the T-type Ca^{2+} channel isoform deduced from INS-1 and α_1G, a section of Sprague-Dawley rat genomic DNA sequence containing the introns and exons between 4845 and 5256 was identified.
20 As shown in Fig. 1B, an exon was found that encodes the α_1G fragment SKEKQMA (SEQ ID NO:5) as well as an exon that encodes fragment 4869-4922 of the INS-1 variant. This region also contains 8.5 kilobases (kb) of intron sequence. Thus, the T-type Ca^{2+} channel α_1 subunit cloned
25 from INS-1 and α_1G are alternative splice isoforms of the same gene.

The genomic DNA sequence was also used to examine the two nucleotide discrepancy between the α_1G cDNA and the isoform cloned from INS-1. The data show that the
30 genomic nucleotide sequence encoding amino acid 1667 is GGC (glycine), which is the same as the cDNA of α_1 subunit cloned from INS-1 and the corresponding residue in α_1H, but is different from α_1G (GCG, alanine). Also of note, there are nine additional single amino acid substitutions

in the isoform deduced from INS-1 as compared to the $\alpha_1 G$. Six correspond to the amino acids found in the analogous position of $\alpha_2 H$: cysteine 1088, glycine 1667, alanine 1700, aspartic acid 1735, threonine 1812, and leucine 1813.

In regard to tissue distribution of T-type Ca^{2+} channels deduced from β-cells and from neurons, expression of the β-cell T-type Ca^{2+} channel was found in rat brain, heart and kidney, but was absent from liver. Both $\alpha_1 G$ and the splice form were detected in rat islets and INS-1 cell preparations using RT-PCR. No $\alpha_1 H$ was detected.

Functional expression of the T-type Ca^{2+} channels deduced from β-cells has been conducted in Xenopus oocytes using a double-electrode voltage-clamp method. In a solution containing 40 mM Ca^{2+}, a family of current traces representing T-type Ca^{2+} current characteristics were obtained (Fig. 2A). The current slowly activated at -40 mV and peaked at -10 mV. The analysis of time constants of activation and inactivation are shown in Fig. 2B. The voltage-dependent activation (Fig. 2C) and steady-state inactivation (Fig. 2D) were fitted with Boltzmann equation. The calculated $V_{1/2}$'s were -23.8 mV and -45.6 mV for activation and inactivation, respectively; and k 's were 5.3 and -6.0 for activation and inactivation, respectively.

The nucleotide cDNA (SEQ ID NO:1) and amino acid (SEQ ID NO:2) sequences of rat pancreatic T-type calcium channel were determined. SEQ ID NO:3 is the nucleotide sequence beyond the coding region, while SEQ ID NO:4 includes SEQ ID NO:2.

EXAMPLE II

Characterization of the T type Calcium Channel
in Relation to Diabetes

Glucose stimulated insulin release is Ca^{2+} dependent
5 process, involving closure of the ATP-sensitive potassium
channels, depolarization and opening of the voltage-
dependent Ca^{2+} channels. At glucose concentrations below
3 mM, which do not elicit insulin secretion, β-cells are
electrically silent with a resting membrane potential of
10 about -70 mV. Raising external glucose produces a slow
depolarization, the extend dependent upon the glucose
concentration. At glucose levels which elicit insulin
release (>7 mM) depolarization is sufficient to reach the
threshold potential (-50 mV) at which electrical activity
15 is initiated.

A simple model for glucose-stimulated insulin
secretion is summarized in Fig. 12. The resting membrane
potential of β-cells is principally determined by the
activity of the K-ATP channel. When plasma glucose
20 rises, its uptake and rate of metabolism by β-cells are
stimulated. As a consequence, the intracellular ATP (or
ATP:ADP ratio) increases which leads to the closure of K-
ATP channels and membrane depolarization. This results
in the activation of voltage dependent Ca^{2+} channels (T-
25 type and L-type) and the initiation of electrical
activity. The increased calcium influx leads to a rise
in $[Ca^{2+}]_i$ and consequently insulin secretion.

Rat and human pancreatic β-cells are equipped with
L-type and T-type Ca^{2+} channels. The physiological
30 function of T-type Ca^{2+} channels in β-cells insulin-
secretion has been demonstrated. These channels
facilitate exocytosis by enhancing electrical activity in
these cells. L-type and T-type Ca^{2+} channels, under
normal conditions, work in concert promoting the rise in

$[Ca^{2+}]_i$ during glucose-stimulated insulin secretion. In
β-cells, over-expressed T-type Ca^{2+} channels are, at least
in part, responsible for the hyper-responsiveness of
insulin secretion to non-glucose depolarizing stimuli in
5 GK rat, and in rat with NIDDM induced by neonatal
injection of streptozotocin. However, over-expressed T-
type calcium channels over time will ultimately lead to
an elevation of basal Ca^{2+} through its window current
properties. Therefore, there is a dual effect of T-type
10 Ca^{2+} channels in β-cells depending upon channel number and
membrane potential.

Pharmacologically antagonizing T-type calcium
channels is an appropriate treatment protocol for
alleviating both insulin resistance and enhancement of
15 insulin secretion in NIDDM patients.

NIDDM pathogenesis is complex and the disease
progression occurs in phases. An enhanced β-cell
responsiveness provokes and initiates the disease
process. It is unclear as to what the actual enhanced
20 activity is and what the triggering mechanisms are for
this first phase. It may be an increased secretory
response or an increase in β-cell mass. However, there
is clearly an enhancement of β-cell activity detected by
both basal and postprandial elevated insulin levels
25 denoted as hyperinsulinemia. Consequently, a resulting
insulin resistance occurs, phase II, particularly in
insulin responsive tissues (muscle, liver, kidney, fat)
that function to reduce glucose levels in the blood. A
decrease in insulin sensitivity will account for an
30 increase in blood glucose, causing the β-cells to secrete
even more insulin to compensate and because of this
vicious cycle, full blown NIDDM, marked by an inevitable
defect in insulin release, hyperglycemia and insulin

resistance, will characterize the final stage of the disease process.

Each phase of the disease may be characterized by an alteration in $[Ca^{2+}]_i$, and each phase can be treated by a T-type calcium channel antagonist. The electrical β-cell is equipped with two types of voltage-dependent calcium channels, L-type and T-type calcium channels. L-type calcium channels, activated at high voltages, having large unitary conductance, and dihydropyridine-sensitive, are considered the major pipeline for calcium influx into the β-cell (especially at high voltage depolarization). T-type calcium channels, activated at low voltages, with small unitary conductance, and dihydropyridine-insensitive, are important for maintaining basal $[Ca^{2+}]_i$ (Fig. 8), as well as enhancing electrical activity during cell depolarization. T-type calcium channels normally facilitate insulin secretion in β-cells by enhancing cell electrical activity. This modulatory function of T-type calcium channels in insulin secretion is significant during phase I prior to onset of diabetes. Antagonizing these T-type calcium channels will decrease β-cell hyper-responsiveness and consequent hyperinsulinemia arresting the pathogenic pathways that lead to NIDDM.

If hyperinsulinemia and associated insulin resistance has already occurred, a T-type calcium channel blocker is still the appropriate treatment protocol. The insulin responsive tissues, those that are primarily responsible for taking up glucose for re-establishing euglycemia, have elevated basal $[Ca^{2+}]_i$ during hyperinsulinemic conditions. Indeed, it is the elevated basal $[Ca^{2+}]_i$ that precipitates the decrease in insulin sensitivity of these tissues and it is now known that most of these insulin responsive tissues express T-type calcium channels. A T-type calcium channel blocker will

reduce the basal $[Ca^{2+}]_i$ and alleviate the decreased insulin sensitivity.

Once NIDDM has manifested, it is characterized by altered glucose metabolism, a result of abnormal glucose stimulus-secretion responsiveness of β-cells. β-cell desensitization to glucose is the principal secretory defect of NIDDM. L-type and T-type calcium channels, under normal conditions, work in concert promoting the rise in $[Ca^{2+}]_i$ during glucose-stimulated insulin secretion. In NIDDM, this partnership is broken and the necessary rise in $[Ca^{2+}]_i$ for insulin secretion is compromised.

The data herein indicates that L-type calcium channels are finely regulated by basal calcium levels (Figs. 9A-9D). A very small rise in basal calcium will substantially decrease the L-type calcium current and severely reduce the depolarization-induced rise in $[Ca^{2+}]_i$ (Figs. 10 and 11). The data herein also suggests that T-type calcium channels are a primary regulator of resting basal $[Ca^{2+}]_i$ in β-cells. Furthermore, the negative feedback regulation of T-type calcium channels by elevated $[Ca^{2+}]_i$ is absent (Figs. 9A-9D). It is under circumstances of enhanced T-type calcium current activity as seen in the GK rat model of NIDDM and in the neonate streptozotocin-induced diabetes model, that basal $[Ca^{2+}]_i$ is elevated, and a defect in the glucose-stimulated insulin secretion is observed. Simply reducing the basal calcium influx by pharmacological intervention, in situations of enhanced T-type calcium channel expression, may reduce basal $[Ca^{2+}]_i$ in β-cells (Fig. 8) and alleviate the $[Ca^{2+}]_i$-induced inhibition of L-type calcium channels.

There is a clear link between $[Ca^{2+}]_i$ and diabetes. A primary abnormality in $[Ca^{2+}]_i$ handling by cells is the defect initiating parallel impairments in insulin

secretion and insulin action, as well as initiating
diabetic complications. Consequent metabolic
derangements may further aggravate alterations in $[Ca^{2+}]_i$
homeostasis, creating a relentless cycle leading to

5 progressive deterioration in the overall health of the
diabetic patient. Pharmacological agents that regulate
$[Ca^{2+}]_i$ homeostasis are thus appropriate therapeutic
measures. The use of T-type calcium channel blockers
will thus effectively treat and perhaps cure diabetes

10 mellitus.

EXAMPLE III

Pharmacology of Mibefradil Action

It has been shown that mibefradil has a potent
15 inhibitory effect on T-type Ca^{2+} current in vascular
smooth muscle cells. The data herein demonstrates that,
in convention whole cell patch clamp configuration,
mibefradil also blocks T-type Ca^{2+} current in pancreatic
β-cells. Mibefradil (1 μM) had been administered in the
20 recording chamber at time zero (Fig. 13), the control (no
drug) showed "run down". This figure shows that T-type
Ca^{2+} current is more sensitive to mibefradil than the L-
type Ca^{2+} current in pancreatic β-cells.

The blockade of T-type Ca^{2+} channels in β-cells with
25 mibefradil is reversible. Fig. 14 demonstrates the
reversibility of blockade of T-type Ca^{2+} currents by
mibefradil. In these experiments, a very little volume
of mibefradil or $NiCl_2$ was delivered near the recording
cell. The drug then diffused away from the cell. The
30 final concentration in the chamber was 1 nM. This
experiment shows the inhibitory effect of mibefradil on
T-type Ca^{2+} current in pancreatic β-cells results from
reversible interaction between the drug and the channel
protein.

In β-cells, T-type Ca^{2+} channels could mediate a small, but sustained, Ca^{2+} influx by means of their unique "window" current at voltages near resting membrane potentials. Like other voltage-regulated channels, T-

5   type Ca^{2+} channels are opened and closed depending upon the potentials across the cell membranes. This voltage dependency is illustrated in Fig. 15. The activation and inactivation curves represent the percentage of the channels in either open or closed states over a range of

10 voltages. Unlike most of the voltage-dependent Na^+ channels or L-type Ca^{2+} channels, the activation and inactivation curves of T-type Ca^{2+} channels overlap at the certain range of low voltages (i.e. window). In other words, there is a small portion of T-type Ca^{2+} channels

15 that stay in non-inactivated states in this voltage range. The data in Fig. 15 was obtained from experiments conducted under 10 mM external Ca^{2+} condition, which shifted the window current about 10 mV toward positive voltage due to the surface charge effects of divalent

20 ions on the channels.

The existence of a window current provides a negative feedback regulation of $[Ca^{2+}]_i$ in β-cells. When cells are under an unhealthy condition, they may be slightly depolarized to activate window current, which

25 elevates the basal $[Ca^{2+}]_i$ to protect the cells from further Ca^{2+} influx through the L-type Ca^{2+} channels. This process is reversible if the membrane potential is reset to the normal resting potential (-70 mV).

30 Mibefradil regulates basal $[Ca^{2+}]_i$ in pancreatic β-cells:

The data herein demonstrates the roles of T-type calcium currents in modulating basal $[Ca^{2+}]_i$ in INS-1 cells (Fig. 8). $[Ca^{2+}]_i$ was directly measured by the ratio of fluorescence excitations at Ca^{2+}-bound (380 nm)

to unbound (340 nm), and then the ratio was converted to
the calcium concentration. The bath solution contained
10 mM NaCl, 4 mM KCl, 2 mM $CaCl_2$, and 2 mM $MgCl_2$. In a
single cell exhibiting fluctuating basal $[Ca^{2+}]_i$ with an
5   average value near 150 nM, administering 1 μM mibefradil
into the chamber immediately lowered the basal calcium.
This data shows the T-type calcium currents participate
in regulating the mean basal $[Ca^{2+}]_i$ in cultured β-cells.

10   Mibefradil regulates basal insulin secretion:

        The activation of T-type Ca^{2+} channel at low voltage
near the resting membrane potential of pancreatic β-cells
suggests that the channels are responsible for the Ca^{2+}
influx required for insulin secretion under non-stimulus
15   conditions. The NIT-1 cell line was chosen to
demonstrate the effect of mibefradil on the basal insulin
secretion. NIT-1 is a cell line derived from the β-cell
of non-obese-diabetic mouse. This cell line expressed
high levels of T-type Ca^{2+} current. The data herein shows
20   that 5 μM mibefradil reduced the basal insulin secretion
to less than 40% of control (Fig. 17), indicating this
drug is able to lower the high basal insulin secretion
level seen during the earlier stage of NIDDM.

25   Spontaneous elevation of $[Ca^{2+}]_i$:

        To demonstrate that T-type Ca^{2+} channels play an
important role in calcium entry under non-stimulatory
conditions, and therefore regulate basal $[Ca^{2+}]_i$,
spontaneous elevations of intracellular free calcium
30   concentration was detected with the Fluo-3 AM fluorescent
imaging method. NIT-1 cells were cultured in medium
containing 3.3 mM glucose and preloaded with 2.5 μM Fluo-
3 AM. The numbers of spontaneous calcium elevated cells
were counted and compared to the total cells being used

for a 10 minute observation period. 10 μM NiCl$_2$ inhibited 90% of spontaneous elevation of basal Ca^{2+}.

The cellular mechanism of the spontaneous elevation of intracellular Ca^{2+} was investigated with the epi-

5 fluorescence measurement method. Some INS-1 cells were observed to exhibit transient spontaneous elevations of [Ca^{2+}]$_i$, "Calcium spikes", under non-stimulatory conditions. The Role of T-type Ca^{2+} channels in this spontaneous process was examined as well. In a single

10 cell with spontaneous calcium spike activity (Fig. 17), NiCl$_2$ (30 μM) reduced the frequency of spontaneous calcium spikes immediately. This result suggests that either the T-type Ca^{2+} channels alone or together with the L-type Ca^{2+} channels are responsible for the transient spontaneous

15 elevation of [Ca^{2+}]$_i$, under conditions where no glucose is present. These spontaneous calcium spikes may contribute to basal insulin secretion and control of basal [Ca^{2+}]$_i$.

However, neither mibefradil nor NiCl$_2$ exhibited their effect on basal [Ca^{2+}]$_i$ in all of the β-cells. It was

20 observed that only those cells which had relatively higher initial basal [Ca^{2+}]$_i$ will respond to the T-type Ca^{2+} channel antagonists (Fig. 18). Whereas those cells with lower initial basal [Ca^{2+}]$_i$ had no or less response to the T type Ca^{2+} channel antagonists. This result

25 indicates that T type Ca^{2+} channel antagonists may selectively act on the cells with high basal [Ca^{2+}]$_i$ and bring it back to normal, by inhibiting the window current.

30

EXAMPLE IV
Action on Pancreatic β-cells

T type Ca^{2+} may play two pathological roles in NIDDM. At the earlier stage, the NIDDM patients exhibit

hyperinsulinemia and β-cell hyperexcitability. This may, at least in part, be due to increased activity of T type Ca^{2+} channel in β-cells. At the more developed NIDDM stage, over-expressed T type Ca^{2+} channel and membrane

5   depolarization resulted from reduced generation of ATP, and may set up a window current in β-cells that causes chronic elevation of basal Ca^{2+} in the β-cells. The elevated basal Ca^{2+} will reduce the L-type Ca^{2+} activity and glucose induced insulin secretion.

10   It has been shown that mibefradil prevented and reversed development of hyperinsulinemia in rat. This result indicates this drug is a valuable candidate for the treatment of earlier stage NIDDM or for preventing NIDDM in the potential patients.

15   A series of experiments were conducted with INS-1 cells to show that T type Ca^{2+} facilitated insulin secretion by enhancing the general excitability of pancreatic β-cells. Particularly, activation of T type Ca^{2+} channels will increase the firing frequency of the

20   depolarizing spikes mediated by opening L type Ca^{2+} channels (Fig. 19A). Activation of T type Ca^{2+} channel will also decrease the time of developing action potential elicited by up-threshold depolarizations (Fig. 19B).

25   To further establish that T type Ca^{2+} current enhances β-cell excitability, 100 μM $NiCl_2$ was administered to effectively block T type Ca^{2+} channels. In contrast to control experiments, $NiCl_2$ caused a delay in the onset of an action potential and a decrease in

30   number of action potentials.

To directly demonstrate the role of T type Ca^{2+} current in glucose-induced insulin secretion, INS-1 cells were incubated with 11.1 mM glucose and variable concentrations of $NiCl_2$, and insulin release was measured.

$NiCl_2$ reduced insulin secretion in a dose-dependent manner
(Fig. 20A). On the other hand, clonal insulin secreting
cells (HIT-T15, which did not consistently exhibit T type
Ca^{2+} current) were not affected by $NiCl_2$ (Fig. 20B).

5   These results show that T type Ca^{2+} channels play an
important role in β-cell excitability and antagonists of
T type Ca^{2+} channels (such an $NiCl_2$) will effectively
reduce the excitability of β-cells.

       Although T type Ca^{2+} channels facilitate insulin
10 secretion by enhancing general excitability of β-cells,
the function of T type Ca^{2+} channels is a doubled-edged
sword. Under the condition of over-expressed T type Ca^{2+}
channel in β-cells, the function of the window current
will become dominant and result in an elevation of basal
15 Ca^{2+}. High $[Ca^{2+}]_i$ may cause impairment of insulin release
by inactivating L type Ca^{2+} channels.

L-type Ca^{2+} channels are partially inactivated by $[Ca^{2+}]_i$
in non-stimulus condition in β-cells:

20   Upon establishment of a whole-cell patch, within the
first five minutes, the L type Ca^{2+} current "runs-up", as
the magnitude of the peak current increases over time in
INS-1 cells (Fig. 21). This phenomenon is a universal
feature in these cells under the recording conditions
25 used. The pipette solutions contained no ATP but did
contain high concentrations of the calcium chelating
agents BAPTA and EGTA. When the pipette solution
contained high Ca^{2+}, this run-up does not occur. Instead,
a rapid run down occurs. The "run-up" phenomenon is
30 likely due to calcium chelation inside the cells. T type
Ca^{2+} currents do not show this effect.

Intracellular perfusion patch clamp experiments demonstrated that basal $[Ca^{2+}]_i$ regulates L type Ca^{2+} current amplitude in INS-1 cells:

Intracellular perfusion of a solution containing

5   high Ca^{2+} (Fig. 9A) causes a substantial reduction in the L type Ca^{2+} current. L type Ca^{2+} currents were elicited by a voltage step to +10 mV from a holding potential of -80 mV. The $[Ca^{2+}]_i$ was measured directly using fura-2 ratiometric fluorescence. The effect of a high $[Ca^{2+}]_i$

10 (272 nM) on the IV relationship is shown in Fig. 9B. Perfusing in high $[Ca^{2+}]_i$, substantially reduces the high voltage current component, but does not affect the low current component. The high $[Ca^{2+}]_i$ caused a shift in peak current to negative voltages, and Ca^{2+} currents were

15 enhanced at negative voltages. This effect seemed to result in a potentiation of the T type Ca^{2+} current (Fig. 9D). Slow deactivating T type Ca^{2+} currents showed a shift in activation upon perfusion of high $[Ca^{2+}]_i$. This may account for the shift in IV. Various concentrations

20 of $[Ca^{2+}]_i$ regulated the activity of L type Ca^{2+} channels (Fig. 9C). Perfusing a low $[Ca^{2+}]_i$ from an existing high $[Ca^{2+}]_i$ (632 nM to 0 nM) caused an increase in the L type Ca^{2+} current over time, however perfusing in high $[Ca^{2+}]_i$ (0 nM to 272 nM and 0 nM to 632 nM) inhibits the L type

25 Ca^{2+} current over time. The levels of $[Ca^{2+}]_i$ therefore have regulatory effects on both the L type Ca^{2+} current and T type Ca^{2+} current, with $[Ca^{2+}]_i$ having significant feedback regulation on the L type Ca^{2+} current.

30 Effect of basal $[Ca^{2+}]_i$ on Ca^{2+} influx:

The effect of basal $[Ca^{2+}]_i$ on Ca^{2+} influx was examined using the Ca^{2+} dye indicator fura-2 and fluorescence measurements. Voltage-dependent Ca^{2+} influx in a single cell was obtained by perfusion of an

osmotically balanced solution containing 50 mM KCl into the recording chamber. Voltage-dependent increases in $[Ca^{2+}]_i$ occur primarily through nifedipine sensitive Ca^{2+} channels. The resting basal $[Ca^{2+}]_i$ in INS-1 cells was

5 approximately 60-80 nM under the experimental conditions. $[Ca^{2+}]_i$ was determined by a standard curve obtained from a fura-2 calcium imaging kit (Molecule Probes). The empirical K_d obtained for calcium binding to fura-2 in the system was 296 ± 20 nM. When basal $[Ca^{2+}]_i$ remains low,

10 subsequent voltage stimulation with 50 KCl induces rapid and large calcium influx into the cell and these calcium changes are stereotyped upon repetitive stimulation when basal calcium is restored (Fig. 10). In this experiment, following the 50 KCl depolarization, the cell was

15 repolarized by perfusion of the original 5 mM KCl solution. After repolarization, basal $[Ca^{2+}]_i$ slowly reset and then a second 50 KCl depolarization induced a similar $[Ca^{2+}]_i$ transient. When the basal calcium is not allowed to reset, a defect in the second voltage induced

20 calcium transient occurs (Fig. 11). In this experiment, after repolarization, the second depolarization was applied before basal $[Ca^{2+}]_i$ could return to its original value, and thus, the $[Ca^{2+}]_i$ transient is substantially reduced. These findings suggest that basal $[Ca^{2+}]_i$ plays

25 a prominent role in the regulation of voltage dependent Ca^{2+} influx in INS-1 cells. Therefore effectors of basal $[Ca^{2+}]_i$ will have important impact on the amount of calcium that can enter the cell.

30 Streptozotocin induced high basal $[Ca^{2+}]_i$ inhibits KCl stimulated Ca^{2+} influx:

To reiterate the importance of basal $[Ca^{2+}]_i$ on voltage stimulated Ca^{2+} influx, basal $[Ca^{2+}]_i$ in INS-1 cells was artificially enhanced by pretreating the cells

with the toxicant, streptozotocin. Though it is know that streptozotocin induces DNA strand breaks, it has also been shown to induce Ca^{2+} channel activity in β-cells. The data shows that pretreating cells with 5 mM

5 streptozotocin for 1 hour, followed by 3 hour recovery, causes a two-fold increase in basal calcium (Fig. 22). These cells when stimulated by 50 KCl had reduced calcium influx compared to control cells.

10 **EXAMPLE V**

Inhibition of T type Calcium Channel

with Mibefradil Metabolite

It has been shown that mibefradil (Ro 40-5967) exerts a selective inhibitory effect on T-type Ca^{2+}

15 currents, although at higher concentrations it can antagonize high voltage-activated Ca^{2+} currents. The action of mibefradil on Ca^{2+} channels is use- and steady state-dependent and the binding site of mibefradil on L-type Ca^{2+} channels is different from that of

20 dihydropyridines. By using conventional whole-cell and perforated patch-clamp, mibefradil is shown to have an inhibitory effect on both T- and L-type Ca^{2+} currents in insulin-secreting cells. However, the effect on L-type Ca^{2+} currents was time-dependent and poorly reversible in

25 perforated patch experiments. Using mass spectrometry it was demonstrated that mibefradil was trapped inside cells and furthermore, a metabolite of mibefradil was detected. Intracellular application of this metabolite selectively blocked the L-type Ca^{2+} current whereas mibefradil exerted

30 no effect. This study shows that mibefradil permeates into cells and is hydrolyzed to a metabolite that blocks L-type Ca^{2+} channels specifically by acting at the inner side of the channel.

Whole-cell patch clamp and a bath perfusion system
were first used to establish the dose-dependent
inhibition of mibefradil on both T- and L-types of Ca^{2+}
currents. The T-type Ca^{2+} current was measured at -30 mV

5 when the membrane was held at -90 mV and the L-type
current was measured at +20 mV when the membrane was held
at -40 mV. The currents were measured twice at each
concentration of mibefradil with 2 min in between
measurements. The dose dependent inhibition of T-type Ca^{2+}

10 current is shown in Fig. 3A. The 50% inhibitory
concentration (IC_{50}) was 865 nM. No time-dependent
inhibition was observed. In contrast, the inhibition of
L-type Ca^{2+} currents could not be fitted with a one-to-one
binding curve (Fig. 3B). Administration of 1 μM

15 mibefradil progressively reduced L-type Ca^{2+} current up to
70% of the beginning amplitude after 10 minutes (n = 4),
which indicated that a more complicated pharmacological
mechanism was involved in the action of mibefradil on the
L-type Ca^{2+} currents.

20 A drug diffusing system was then used to test the
reversibility of the antagonism of T- and L-type Ca^{2+}
currents by mibefradil. Small volumes (approximately 2
μl) of drugs were delivered in close proximity to the
recording cell with a quartz capillary positioned by a

25 micromanipulator. After administration, drugs diffused
throughout the entire recording chamber containing 2 ml
of bath solution. This drug diffusing system was used to
test the reversibility of 30 μM of $NiCl_2$ on the T-type
Ca^{2+} currents (Fig. 4). The amplitude of T-type current

30 was rapidly reduced to 40% and gradually returned to 80%
of the initial level within 3 minutes. Using this system,
it was found that the inhibition of mibefradil on the
T-type Ca^{2+} current was clearly reversible. In contrast,

the inhibition of the L-type Ca^{2+} current was poorly reversible (Fig. 4).

The poor reversibility and time-dependent inhibition of the L-type Ca^{2+} current by mibefradil suggested that
5   this drug may have an accumulation effect over time. This hypothesis was tested by applying a very low dose of mibefradil on cells and recording the L-type Ca^{2+} currents for a long time in the perforated patch-clamp configuration. As shown in Fig. 5A, after 25 minutes of
10   10 nM mibefradil administration, the relative currents were reduced to 70%, whereas the currents remained unchanged for control patches. Incubation of cells with 10 nM mibefradil for two hours resulted in further reduction of current densities as recorded by perforated
15   patches (Fig. 5B). At a concentration of 10 nM, mibefradil exhibited no long-term effect on the T-type Ca^{2+} current.

To test the hypothesis that mibefradil may permeate through the cell membrane to the cytoplasm and be trapped
20   inside cells, the presence of mibefradil was examined in cells pre-incubated with 20 μM of mibefradil using mass spectrometry. After 3 washes, mibefradil (peaked at 496 MW) was still detected in cells (Fig. 6B). The concentration of intracellular mibefradil after one
25   minute incubation was 3.18 $\pm$ 0.78 μM (n = 3). The localization of mibefradil in cells was examined by measuring the concentration of mibefradil in the pellets and supernatants after lysis of the cells. Most of the mibefradil (92%) was detected in the supernatants and 0%
30   was found in the pellets after washing cells with methanol, indicating that mibefradil was trapped in the cytoplasm. In addition, a peak (MW = 423) was detected which represented a hydrolyzed metabolite of mibefradil, Des-methoxyacetyl mibefradil (dm-mibefradil), which is a

major metabolite as documented previously (Wiltshire et al. 1992). By varying the time of pre-incubation, it was found that dm-mibefradil accumulated inside the cells in a time-dependent manner (Fig. 6A). This accumulation is

5   consistent with the concept that dm-mibefradil has lower membrane permeability than its precursor mibefradil.

It was then tested whether or not mibefradil or dm-mibefradil inhibits L- or T-type Ca^{2+} currents from inside of cells. Both L- and T-type currents were

10   measured in the whole-cell patch clamp configuration when 1 μM of mibefradil or dm-mibefradil was included in the pipette solution. As shown in Figs. 7A and 7B, intracellular application of 1 μM mibefradil did not have inhibitory effects on either L-type or T-type Ca^{2+}

15   currents, whereas the same concentration of dm-mibefradil specifically blocked the L-type Ca^{2+} current. As the bath solution contained no drug in this series of experiments, the inhibitory effect of dm-mibefradil is considered to be acting on the inside domain of L-type Ca^{2+} channels.

20   The inhibitory effect of dm-mibefradil on T-type Ca^{2+} currents was similar to the effect of mibefradil when it was applied in the bath solution, suggesting that the methoxyacetyl group of mibefradil does not play a key role in binding to the extracellular receptor site of

25   T-type Ca^{2+} channel protein. However, this methoxyacetyl group is necessary for blocking L-type Ca^{2+} channel from the inside of cells, indicating a modification in the methoxyacetyl group of mibefradil can result in a more selective antagonist of T-type Ca^{2+} channels.

30

EXAMPLE VI

LVA Ca^{2+} Current Mediates Cytokine-Induced
Pancreatic β-cell Death

Insulin-dependent diabetes mellitus is characterized

5   by the selective destruction of pancreatic β-cells.
Chronic treatment with cytokines induced a low voltage-activated (LVA) Ca^{2+} current in mouse β-cells. The
concomitant increase in the basal cytoplasmic free Ca^{2+}
concentration ([Ca^{2+}]$_i$) was associated with DNA

10 fragmentation and cell death. Antagonists of LVA Ca^{2+}
channels prevented this elevation of basal [Ca^{2+}]$_i$ and DNA
fragmentation, and reduced the percentage of cell death.
Exposure to cytokines did not affect the profile of Ca^{2+}
currents or basal [Ca^{2+}]$_i$ in glucagon-secreting α-cells.

15 An increased Ca^{2+} signal through LVA Ca^{2+} channels may thus
be a key feature in cytokine-induced β-cell destruction.

The effects of chronic cytokine treatment on the
voltage-sensitive Ca^{2+} currents in primary cultured mouse
islet cells was examined. After treatment with IL-1β (25

20 U/ml) and IFNγ (300 U/ml) for 6 h, an LVA Ca^{2+} current was
induced in these cells (Fig. 23A). This current was
present in 48% of cytokine-treated mouse islet cells. No
such current was observed when the cells were treated
with either IL-1β or IFNγ alone. Experiments were

25 conducted at different times recording LVA Ca^{2+} currents
induced by cytokines, and the results indicate that no
further increase in current density occurs even after
treatment for 48 h. This LVA current has not been
observed in non-treated cells. The steady state

30 inactivation curve of the cytokine-induced LVA Ca^{2+}
currents displayed a low voltage property (Fig. 23E)
similar to the inactivation curve of the LVA currents in
NOD mouse islets cells. This current was also blocked by
NiCl$_2$ (10 μM; n = 4; Fig. 23F). It has been reported that

low concentration of $NiCl_2$ selectively block LVA current, a profound increase in Ca^{2+} current density was observed over the voltages between -20 and 20 mV. These high voltage-activated Ca^{2+} currents are nifedipine sensitive

5   currents (completely blocked by 10 μM nifedipine), and the increase in this current density is similar to the increased L type Ca^{2+} current density observed after treatment of β-cells with serum from IDDM patients.

As α-cells are more resistant to the toxic effects

10   of cytokines than β-cells, the effects of cytokines on the Ca^{2+} currents in a glucagon-secreting cell line (α-TC1) was also examined. This cell line, like α-cells, is more resistant to the cytotoxic effect of cytokines. Treatment of α-TC1 cells with IL-1β and IFNγ failed to

15   induce LVA Ca^{2+} currents and did not alter the current density (Figs. 23C and 23D). Therefore, the induction of LVA Ca^{2+} currents and increased Ca^{2+} current density observed after chronic treatment with cytokines showed specificity for β-cells.

20   LVA Ca^{2+} channels are activated at low membrane potentials. This unique feature may allow then to regular $[Ca^{2+}]_i$ under nonstimulatory conditions. Indeed, basal $[Ca^{2+}]_i$ in cytokine-treated cells was approximately 3-fold higher than in nontreated cells (Fig. 24A). This

25   increase in basal $[Ca^{2+}]_i$ was blocked by $NiCl_2$ (10 μM), but not by the L type Ca^{2+} channel antagonist, nifedipine (10 μM). Cytokines failed to increase basal $[Ca^{2+}]_i$ in α-TC1 cells (Fig. 24B). These results suggest that Ca^{2+} influx through LVA Ca^{2+} channels is responsible for the cytokine-

30   induced elevation in basal $[Ca^{2+}]_i$ in β-cells.

It has been shown that cytokines induce apoptosis in human pancreatic islet cells. Apoptosis is also the mode of cell death in the development of IDDM in the NOD mouse and in multiple low dose streptozotocin-induced IDDM in

the mouse, and is involved in β-cell destruction. As a marker of apoptosis, DNA fragmentation has been reported to precede β-cell lysis.

β-TC3 cells, a mouse β-cell line, were used to
5 demonstrate the role of LVA Ca^{2+} channels in cytokine-mediated DNA fragmentation. The LVA Ca^{2+} current density was first examined before and after cytokine treatment. The LVA Ca^{2+} current (at $V_m = -30$ mM) in β-TC3 cells was increased from 1.86 ± 0.33 (pA/pF; n = 30) to 3.45 ± 0.47
10 (pA/pF; n = 10) after treatment with cytokines (25 U/ml IL-1β, 100 U/ml IFNγ, and 100 U/ml TNFα) for 25 h. This indicates that the LVA Ca^{2+} current in β-TC3 cells is regulated by cytokines, as seen in mouse islet cells. As shown in Fig. 24, cytokine-induced DNA fragmentation
15 displayed a ladder pattern of oligonucleosomal fragments. The three LVA Ca^{2+} channel blockers, $NiCl_2$, amiloride, and mibefradil, all independently prevented cytokine-induced DNA fragmentation. In contrast, nifedipine had not inhibitory effect on DNA fragmentation induced by
20 cytokines. This experiment has been repeated in β-TC3 cells (n = 2) as well as in NIT-1 cells (n = 3), a β-cell line derived from NOD mice, and the same results were obtained.

The function of LVA Ca^{2+} channels in cytokine-
25 mediated cell death in β-TC3 cells was then examined. Many cells died when the medium contained 25 U/ml IL-1β, 100 U/ml IFNγ, and 100 U/ml TNFα; however, $NiCl_2$ (20 μM) effectively reduced the β-cell killing potency of cytokines in both a time- and dose-dependent manner
30 (Figs. 25A and 25B, respectively). In contrast, nifedipine did not exhibit a protective effect. Similar results were obtained from an experiment conducted in NIT-1 cells with mibefradil, which also reduced β-cell death induced by cytokines. These results demonstrate

that LVA Ca^{2+} channels enhance the vulnerability of β-cells to the cytotoxic effects of cytokines.

Although preferred embodiments have been depicted and described in detail herein, it will be apparent to those skilled in the relevant art that various modifications, additions, substitutions and the like can be made without departing from the spirit of the invention and these are therefore considered to be within the scope of the invention as defined in the claims which follow.

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What Is Claimed Is:

1   1.   An isolated nucleic acid molecule encoding a
2   pancreatic T-type calcium channel.

1   2.   The isolated nucleic acid molecule of claim 1
2   wherein said nucleic acid is deoxyribonucleic acid.

1   3.   The isolated nucleic acid molecule of claim 2
2   wherein said deoxyribonucleic acid is cDNA.

1   4.   The isolated nucleic acid molecule of claim 3
2   wherein said nucleic acid molecule has a nucleotide
3   sequence as shown in SEQ ID NO:1.

1   5.   The isolated nucleic acid molecule of claim 1
2   wherein said nucleic acid molecule encodes an amino acid
3   sequence as shown in SEQ ID NO:2.

1   6.   The isolated nucleic acid molecule of claim 1
2   wherein said nucleic acid is ribonucleic acid.

1   7.   The isolated nucleic acid molecule of claim 6
2   wherein said ribonucleic acid is mRNA.

1   8.   An antisense nucleic acid molecule complementary
2   to at least a portion of the mRNA of claim 7.

1   9.   A cell comprising the antisense nucleic acid
2   molecule of claim 8.

1   10.   An expression vector comprising the antisense
2   nucleic acid molecule of claim 8.

1   11. The expression vector of claim 10 wherein the
2 expression vector is selected from the group consisting
3 of a plasmid and a virus.

1   12. A cell comprising the expression vector of
2 claim 10.

1   13. A method of decreasing expression of a
2 pancreatic T-type calcium channel in a host cell, said
3 method comprising introducing the antisense nucleic acid
4 molecule of claim 8 into the cell, wherein said antisense
5 nucleic acid molecule blocks translation of said mRNA so
6 as to decrease expression of said pancreatic T-type
7 calcium channel in said host cell.

1   14. A ribozyme having a recognition sequence
2 complementary to a portion of the mRNA of claim 7.

1   15. A cell comprising the ribozyme of claim 14.

1   16. An expression vector comprising the ribozyme of
2 claim 14.

1   17. The expression vector of claim 16 wherein the
2 expression vector is selected from the group consisting
3 of a plasmid and a virus.

1   18. A cell comprising the expression vector of
2 claim 16.

1   19. A method of decreasing expression of a
2 pancreatic T-type calcium channel in a host cell, said
3 method comprising introducing the ribozyme of claim 14
4 into the cell, wherein expression of said ribozyme in

5   said cell results in decreased expression of said

6   pancreatic T-type calcium channel in said cell.

1   20. A cell comprising the nucleic acid molecule of

2   claim 1.

1   21. An expression vector comprising the nucleic

2   acid molecule of claim 1.

1   22. The expression vector of claim 21 wherein said

2   expression vector is selected from the group consisting

3   of a plasmid and a virus.

1   23. A cell comprising the expression vector of

2   claim 21.

1   24. A method of increasing expression of pancreatic

2   T-type calcium channel in a host cell, said method

3   comprising:

4   introducing the nucleic acid molecule of

5   claim 1 into the cell; and

6   allowing said cell to express said nucleic acid

7   molecule resulting in the production of pancreatic T-type

8   calcium channel in said cell.

1   25. A method of screening a substance for the

2   ability of the substance to modify T-type calcium channel

3   function, said method comprising:

4   introducing the nucleic acid molecule of claim 1

5   into a host cell;

6   expressing said pancreatic T-type calcium channel

7   encoded by said nucleic acid molecule in the host cell;

8   exposing the cell to a substance; and

9 evaluating the exposed cell to determine if the

10 substance modifies the function of the T-type calcium

11 channel.

1   26. The method of claim 25 wherein said evaluation

2 comprises monitoring the expression of T-type calcium

3 channel.

1   27. A method of obtaining DNA encoding a pancreatic

2 T-type calcium channel, said method comprising:

3   selecting a DNA molecule encoding a pancreatic T-

4 type calcium channel, said DNA molecule having a

5 nucleotide sequence as shown in SEQ ID NO:1;

6   designing an oligonucleotide probe for a pancreatic

7 T-type calcium channel based on SEQ ID NO:1;

8   probing a genomic or cDNA library of an organism

9 with the oligonucleotide probe; and

10   obtaining clones from said library that are

11 recognized by said oligonucleotide probe, so as to obtain

12 DNA encoding a pancreatic T-type calcium channel.

1   28. A method of obtaining DNA encoding a pancreatic

2 T-type calcium channel, said method comprising:

3   selecting a DNA molecule encoding a pancreatic

4 T-type calcium channel, said DNA molecule having a

5 nucleotide sequence as shown in SEQ ID NO:1;

6   designing degenerate oligonucleotide primers

7 based on SEQ ID NO:1; and

8   utilizing said oligonucleotide primers in a

9 polymerase chain reaction on a DNA sample to identify

10 homologous DNA encoding a pancreatic T-type calcium

11 channel in said sample.

1   29. An isolated nucleic acid molecule encoding a
2 pancreatic T-type calcium channel, said nucleic acid
3 molecule encoding a first amino acid sequence having at
4 least 90% amino acid identity to a second amino acid
5 sequence, said second amino acid sequence as shown in SEQ
6 ID NO:2.

1   30. A DNA oligomer capable of hybridizing to the
2 nucleic acid molecule of claim 1.

1   31. A method of detecting presence of a pancreatic
2 T-type calcium channel in a sample, said method
3 comprising:
4   contacting a sample with the DNA oligomer of claim
5 30, wherein said DNA oligomer hybridizes to any of said
6 pancreatic T-type calcium channel present in said sample,
7 forming a complex therewith; and
8   detecting said complex, thereby detecting presence
9 of a pancreatic T-type calcium channel in said sample.

1   32. The method of claim 31 wherein said DNA
2 oligomer is labeled with a detectable marker.

1   33. An isolated pancreatic T-type calcium channel
2 protein.

1   34. The pancreatic T-type calcium channel protein
2 of claim 33 wherein said pancreatic T-type calcium
3 channel protein is encoded by a nucleotide sequence as
4 shown in SEQ ID NO:1.

1   35. The pancreatic T-type calcium channel protein
2 of claim 33 wherein said pancreatic T-type calcium

3   channel protein is encoded by an amino acid sequence as

4   shown in SEQ ID NO:2.

1   36.   An isolated pancreatic T-type calcium channel

2   protein encoded by a first amino acid sequence having at

3   least 90% amino acid identity to a second amino acid

4   sequence, said second amino acid sequence as shown in SEQ

5   ID NO:2.

1   37.   An antibody or fragment thereof specific for

2   the pancreatic T-type calcium channel protein of claim

3   36.

1   38.   The antibody of claim 37 wherein said antibody

2   comprises a monoclonal antibody.

1   39.   The antibody of claim 37 wherein said antibody

2   comprises a polyclonal antibody.

1   40.   A composition comprising the pancreatic T-type

2   calcium channel protein of claim 36 and a compatible

3   carrier.

1   41.   A method of detecting presence of a pancreatic

2   T-type calcium channel protein in a sample, said method

3   comprising:

4   contacting a sample with the antibody or fragment

5   thereof of claim 37, wherein said antibody or fragment

6   thereof binds to any of said pancreatic T-type calcium

7   channel protein present in said sample, forming a complex

8   therewith; and

9   detecting said complex, thereby detecting presence

10 of a pancreatic T-type calcium channel protein in said

11 sample.

1   42. The method of claim 41 wherein said antibody or
2 fragment thereof is labeled with a detectable marker.

1   43. A method of modifying insulin secretion by
2 pancreatic beta cells, the method comprising modifying
3 levels of functional T type calcium channels in the
4 pancreatic beta cells.

1   44. The method of claim 43 wherein modifying levels
2 of functional T type calcium channels comprises modifying
3 T type calcium channel gene expression in the pancreatic
4 beta cells.

1   45. The method of claim 44 wherein modifying T type
2 calcium channel gene expression comprises exposing the
3 pancreatic beta cells to a compound which modifies T type
4 calcium channel gene expression.

1   46. The method of claim 45 wherein the compound is
2 an antisense oligonucleotide targeted to the T type
3 calcium channel gene.

1   47. The method of claim 43 wherein modifying levels
2 of functional T type calcium channel comprises exposing
3 the pancreatic beta cells to an inhibitor of the
4 functional T type calcium channel.

1   48. The method of claim 43 wherein modifying levels
2 of functional T type calcium channel comprises exposing
3 the pancreatic beta cells to a compound which interferes
4 with membrane T type calcium channel formation.

1   49. The method of claim 43 wherein the pancreatic

2 beta cells are present in a subject having type II

3 diabetes.

1   50. A method of treating type II diabetes in a

2 subject, the method comprising administering to the

3 subject an amount of a compound effective to modify

4 levels of functional T type calcium channel in the

5 pancreatic beta cells of the subject.

1   51. The method of claim 50 wherein the compound

2 modifies levels of functional T type calcium channel by

3 modifying T type calcium channel gene expression.

1   52. The method of claim 51 wherein modifying T type

2 calcium channel gene expression comprises exposing the

3 pancreatic beta cells to a compound which modifies T type

4 calcium channel gene expression.

1   53. The method of claim 52 wherein the compound is

2 an antisense oligonucleotide targeted to the T type

3 calcium channel gene.

1   54. The method of claim 50 wherein the compound is

2 an inhibitor of the functional T type calcium channel.

1   55. The method of claim 50 wherein the compound

2 interferes with membrane T type calcium channel

3 formation.

1   56. A method of modifying basal calcium levels in

2 cells, the method comprising modifying levels of

3 functional T type calcium channels in the cells.

1   57.   A method of modifying the action potential of L
2   type calcium channels in cells, the method comprising
3   modifying levels of functional T type calcium channels in
4   the cells.

1   58.   A method of modifying pancreatic beta cell
2   death, the method comprising modifying levels of
3   functional T type calcium channels in the pancreatic beta
4   cells.

1   59.   A method of modifying pancreatic beta cell
2   proliferation, the method comprising modifying levels of
3   functional T type calcium channels in the pancreatic beta
4   cells.

1   60.   A method of modifying calcium influx through L
2   type calcium channels in cells, the method comprising
3   modifying levels of functional T type calcium channels in
4   the cells.

T-TYPE CALCIUM CHANNEL

ABSTRACT OF THE DISCLOSURE

The present invention is directed to isolated

5   nucleic acid molecules encoding pancreatic T-type calcium channels. Expression vectors and host cells comprising the nucleic acid molecules are also provided, as well as methods for increasing or decreasing the expression of pancreatic T-type calcium channel in host cells. The

10   invention further provides a method of screening a substance for the ability of the substance to modify T-type calcium channel function, and a method for isolating other pancreatic T-type calcium channel molecules. DNA oligomers capable of hybridizing to the nucleic acid

15   molecule encoding the pancreatic T-type calcium channel are provided, which can be used to detect pancreatic T-type calcium channel in a sample. An isolated pancreatic T-type calcium channel protein is also provided. Antibodies specific for the protein, and fragments

20   thereof, are provided, as are compositions comprising the protein and a compatible carrier. The subject invention further provides a method of modifying insulin secretion by pancreatic beta cells, a method of treating type II diabetes in a subject, a method of modifying basal

25   calcium levels in cells, a method of modifying the action potential of L type calcium channels in cells, a method of modifying pancreatic beta cell death, a method of modifying pancreatic beta cell proliferation, and a method of modifying calcium influx through L type calcium

30   channels in cells.

Fig. 1A

(1) atgctctccccaccacgggtccccgttgcgtgaggacacctcctctgaggggtctccgctcgccctcttcg
(2) **ATG**ctccccacegggg-tcccccg-ttgcgtgaggacacctcctctgaggggtctccgctcgccctcttcg

(1) gacccccgggggccccgggctggccagagg**ATG**gacgaggaggaggatggagcggggcgccgaggagtcggga
(2) gacccccgggggccccgggctggccagaggatggagcggggcgccgaggagtcggga

(1) cagccccgtagcttcacgcagctcaacgacctgtccgggggcggggcgcaggggccgggg
(2) cagccccgtagcttcacgcagctcaacgacctgtccggggggc-ggcaggggccgggg-
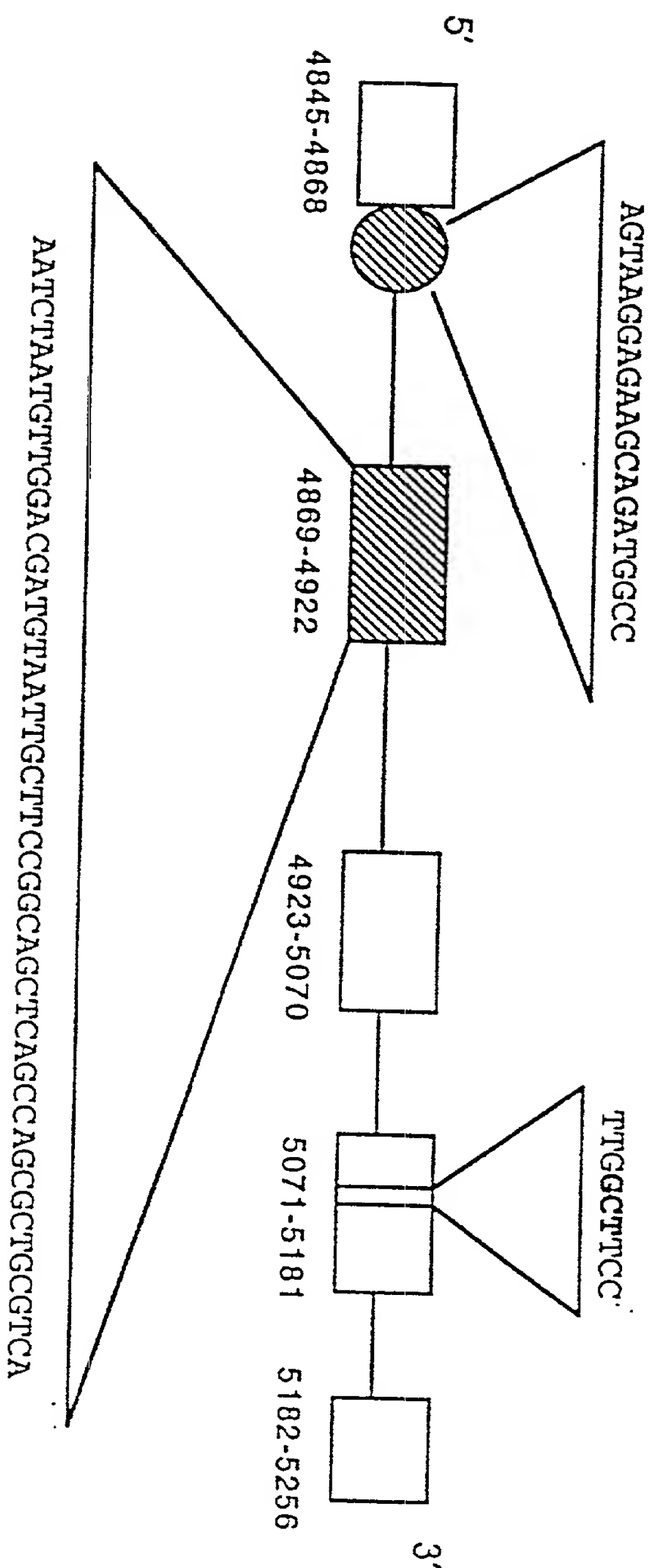
Fig. 1B

5'

4845-4868   4869-4922   4923-5070   5071-5181   5182-5256   3'

AGTAAGGAGAAGCAGATGGCC

TTGGCTTCC

AATCTAATGTTGGACGATGTAATTGCTTCCGGCAGCTCAGCGCTGCGTCA

Fig. 2B

Inactivation τ (ms)

mV

Activation τ (ms)

mV

Fig. 2D

Prepulse (mV)

Relative I (%)

Fig. 2A

200 nA

20 ms

Fig. 2C

Test Potential (mV)

Relative G (%)

Fig. 3A

Fig. 3B

Fig. 4

Fig. 5A

Fig. 5B

Relative Current

Time (min)

pA/pF

Control   Mibefradil

*

Fig. 6B

Fig. 6A

Fig. 7A

Fig. 7B

Fig. 8

Fig. 9A

Fig. 9B

Fig. 9C

Fig. 9D

Fig. 10

Fig. 11

Fig. 12

GLUCOSE

Glucose Metabolism

$\downarrow$

$\updownarrow$ ATP/ADP

$\downarrow$

K-ATP channels close

$\downarrow$

depolarization

$\downarrow$

Ca-channels open

$\downarrow$

$\updownarrow$ [Ca]$_i$

INSULIN SECRETION

Fig. 13

Legend:
- L-Type control
- L-Type Mibefradil
- T-Type Mibefradil

(Relative Current vs. Time (Min.))

Fig. 14

Fig. 15

Fig. 16

Fig. 17

Fig. 18

Fig. 19A

Fig. 19B

Fig. 20A

Fig. 20B

Fig. 21

Fig. 22

Fig. 23A.

Untreated

Cytokine-treated

10 pA

20 ms

Fig. 23B.

Fig. 23C.

Untreated

Cytokine-treated

10 pA

20 ms
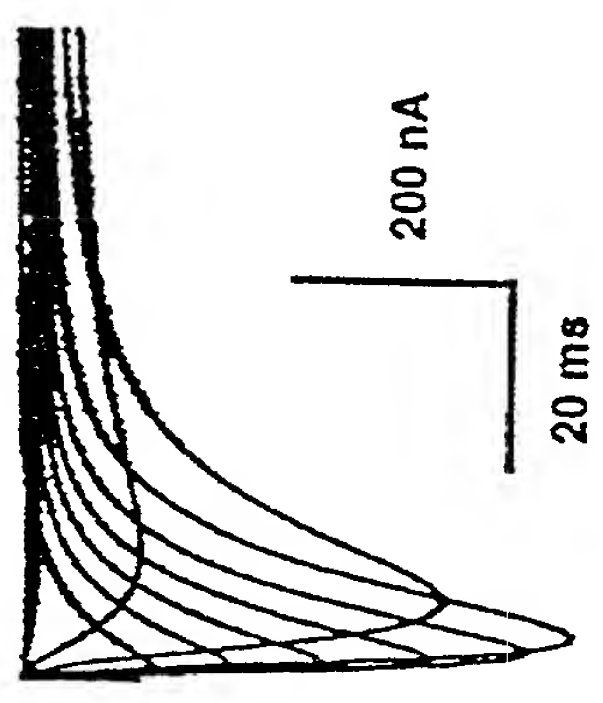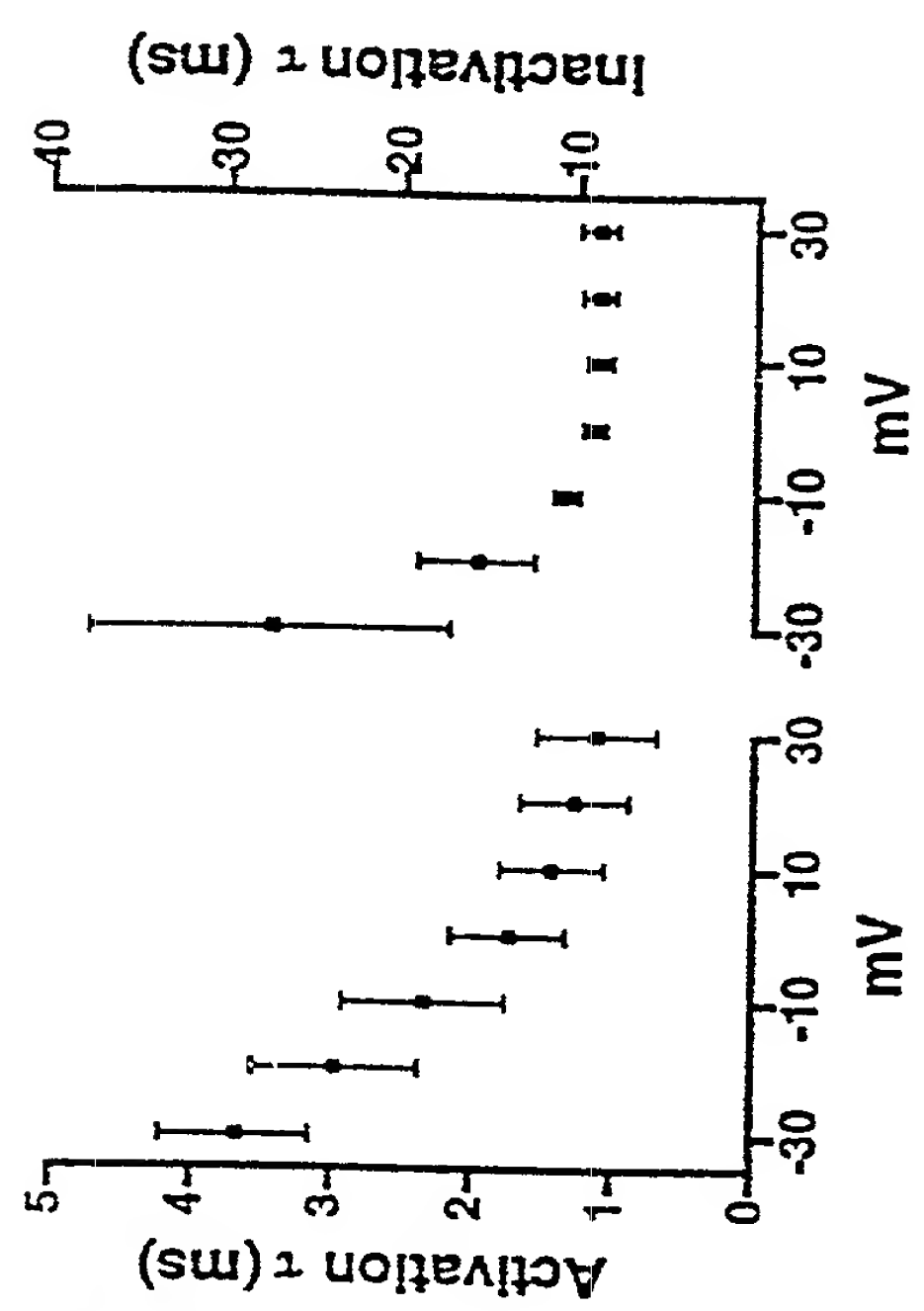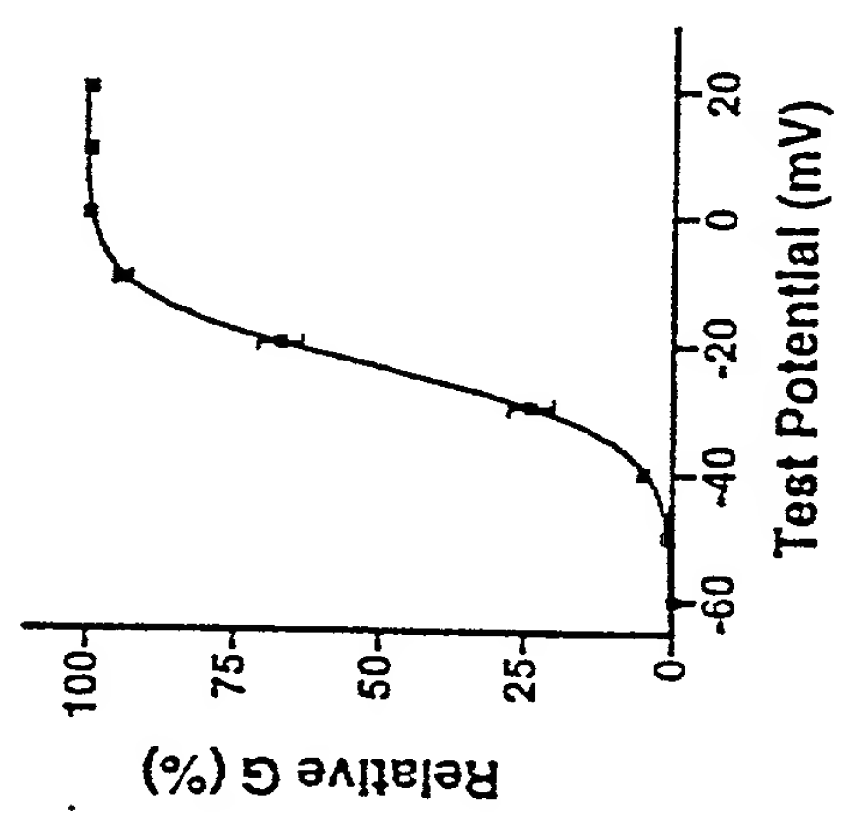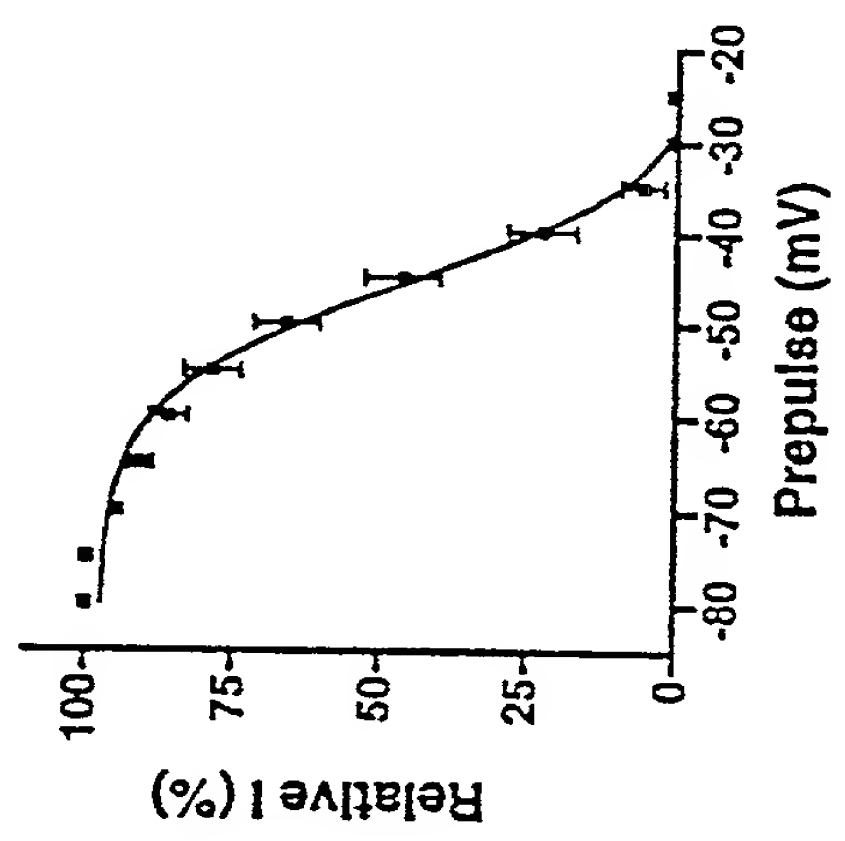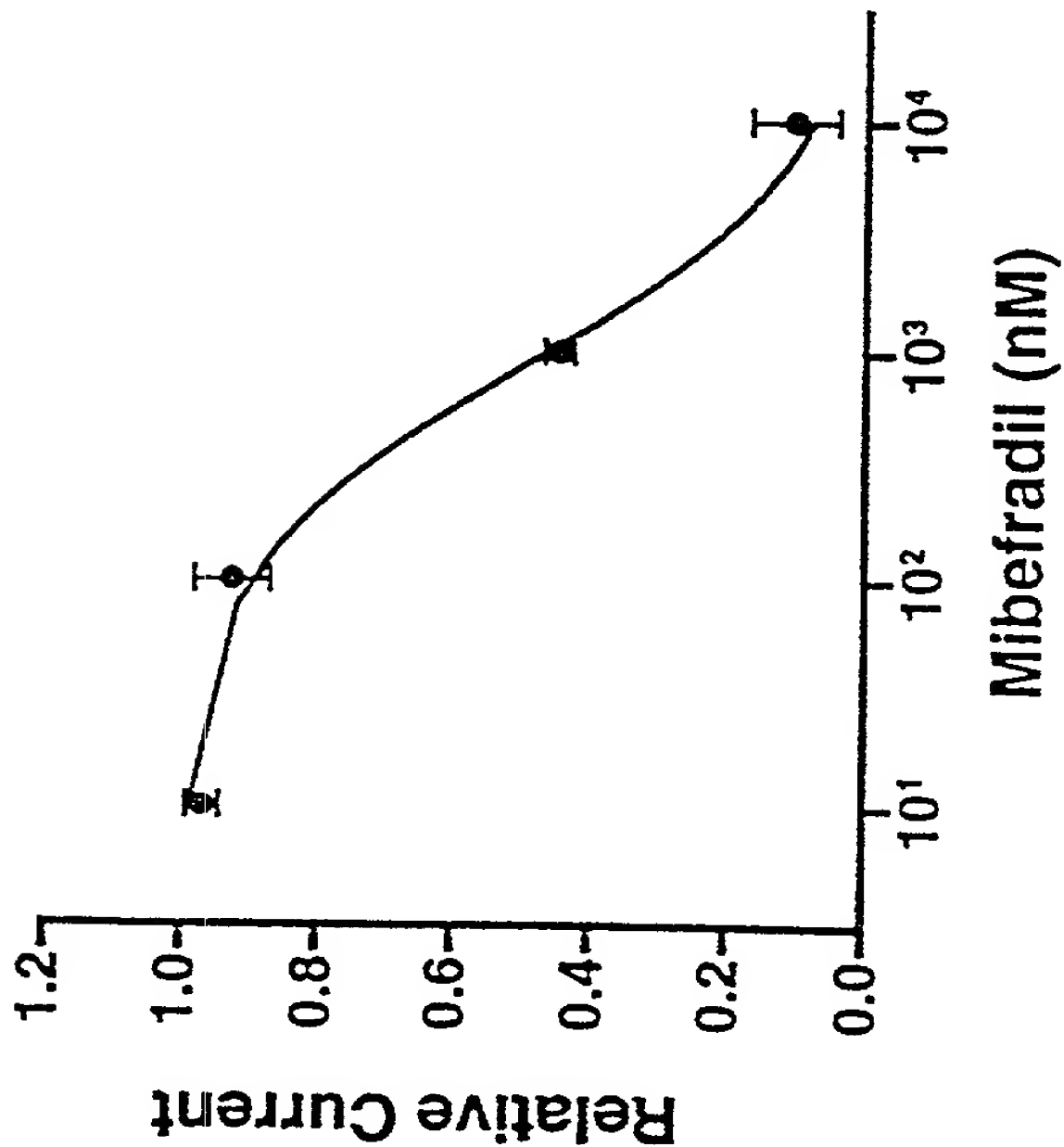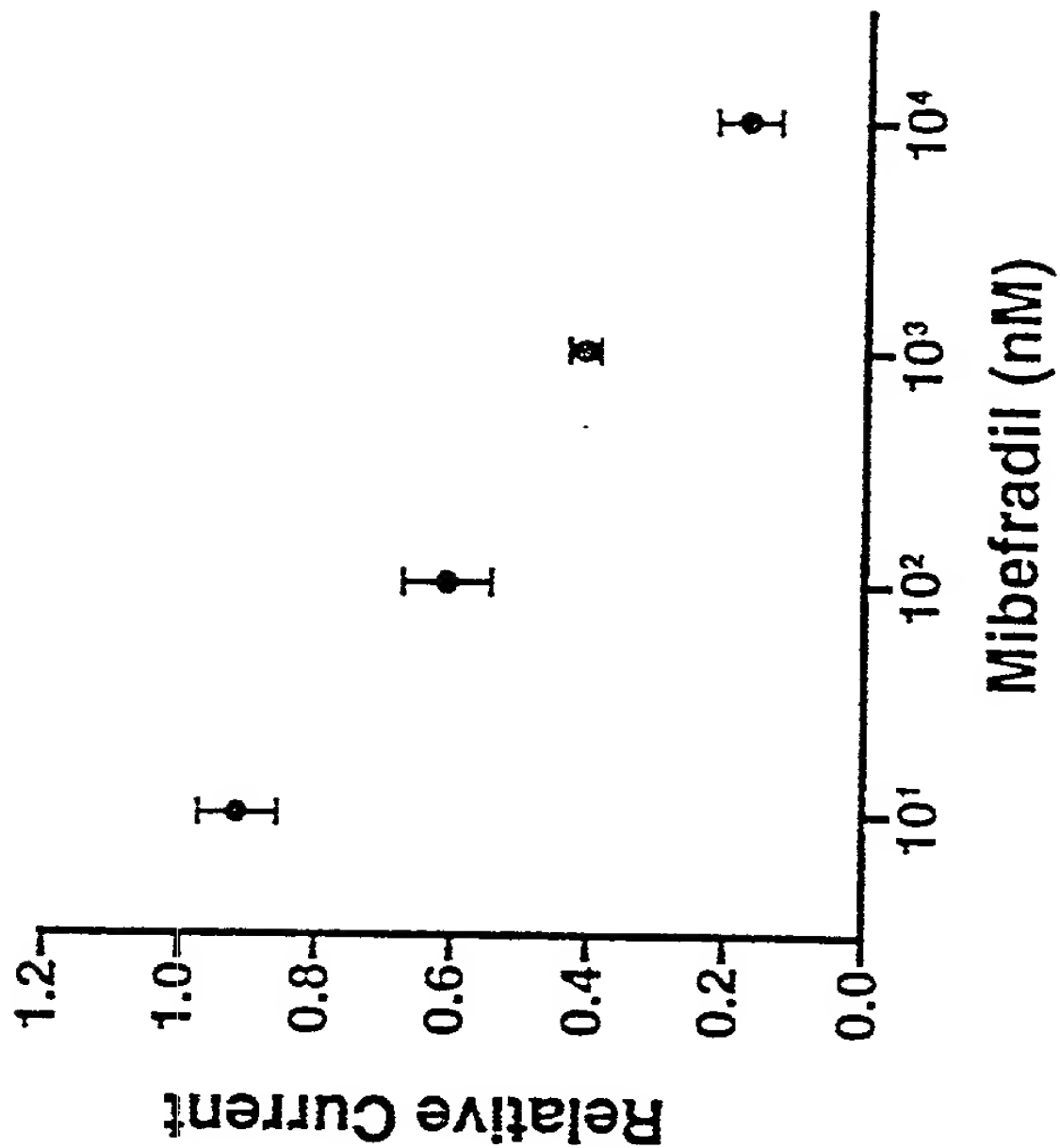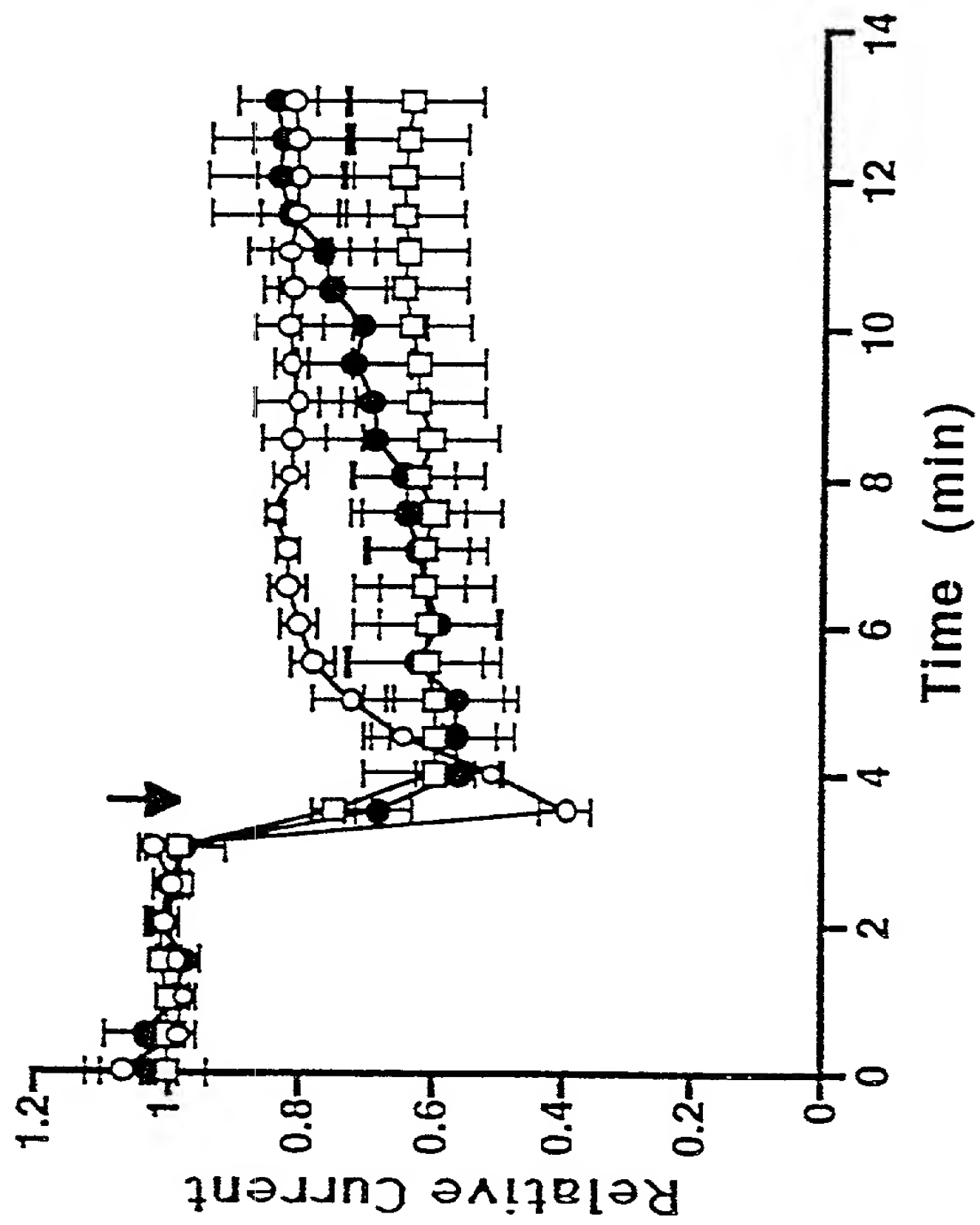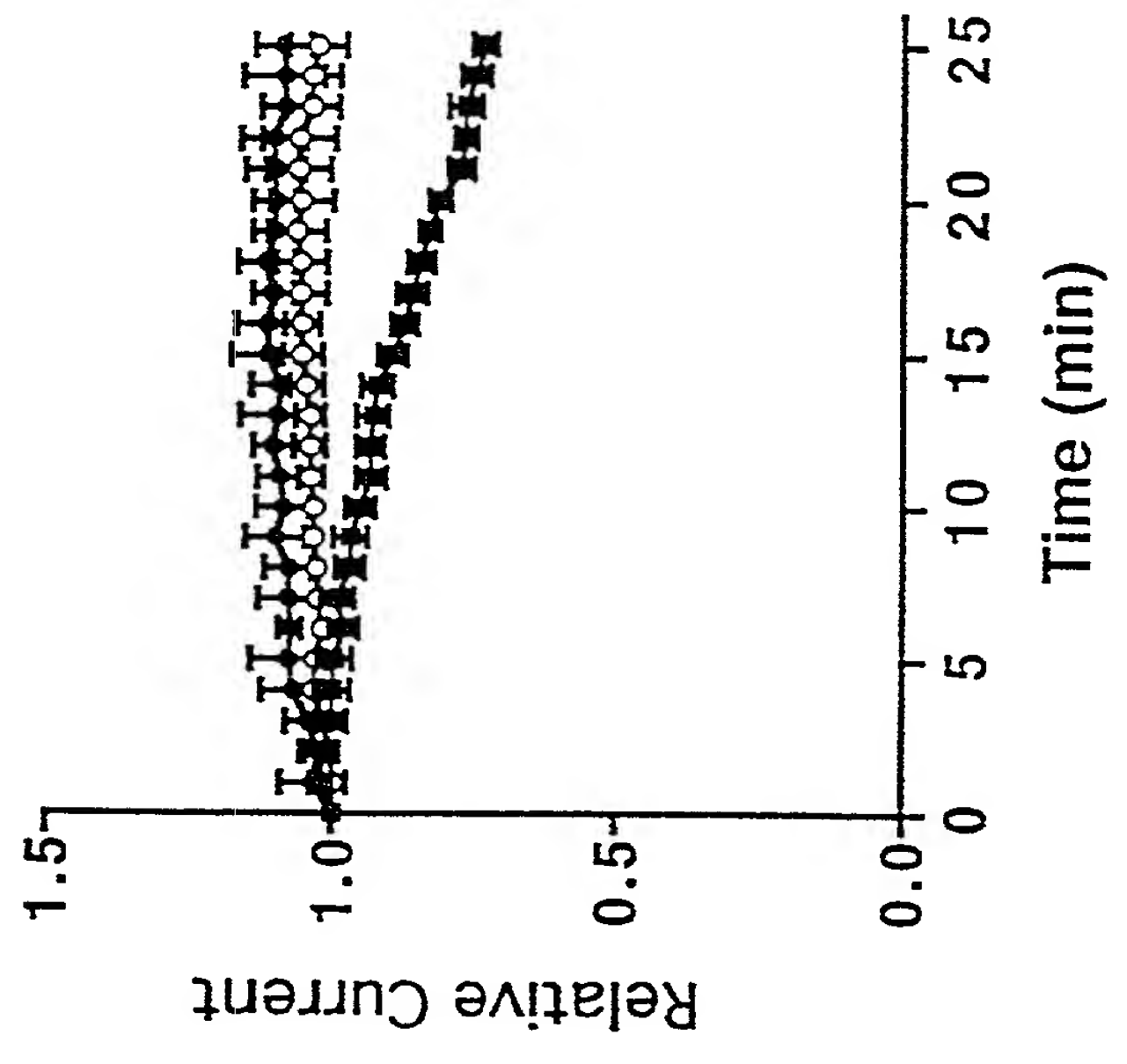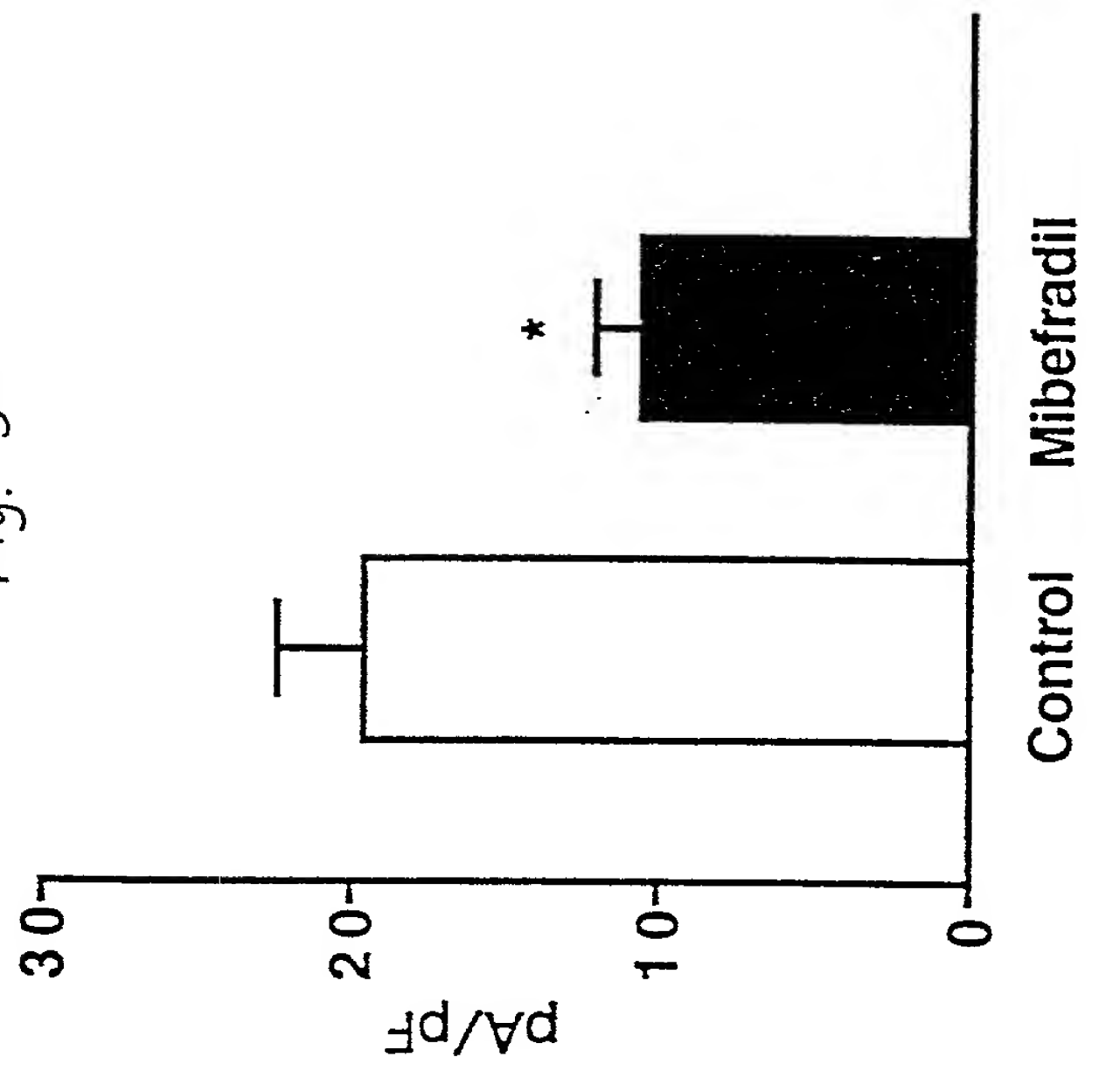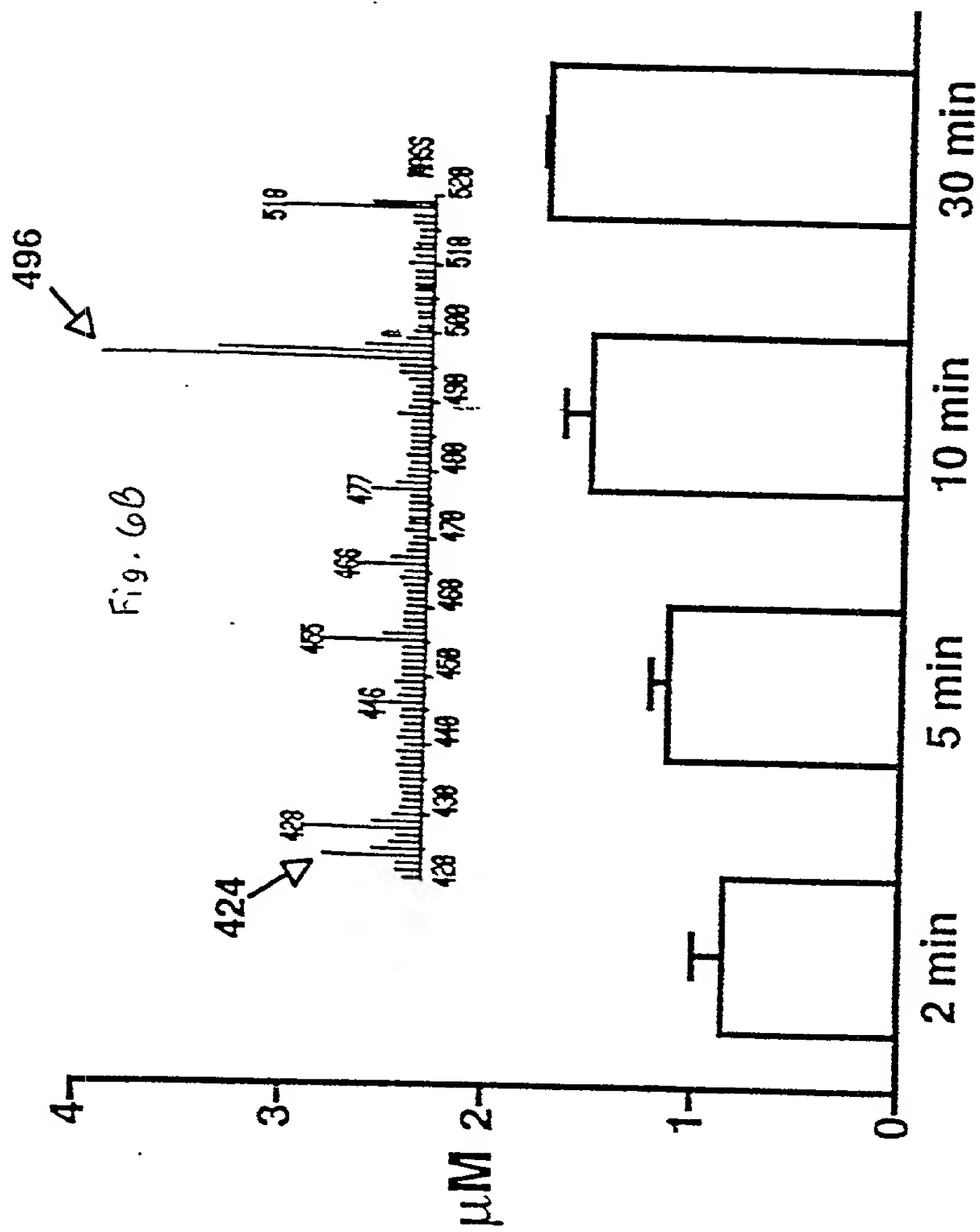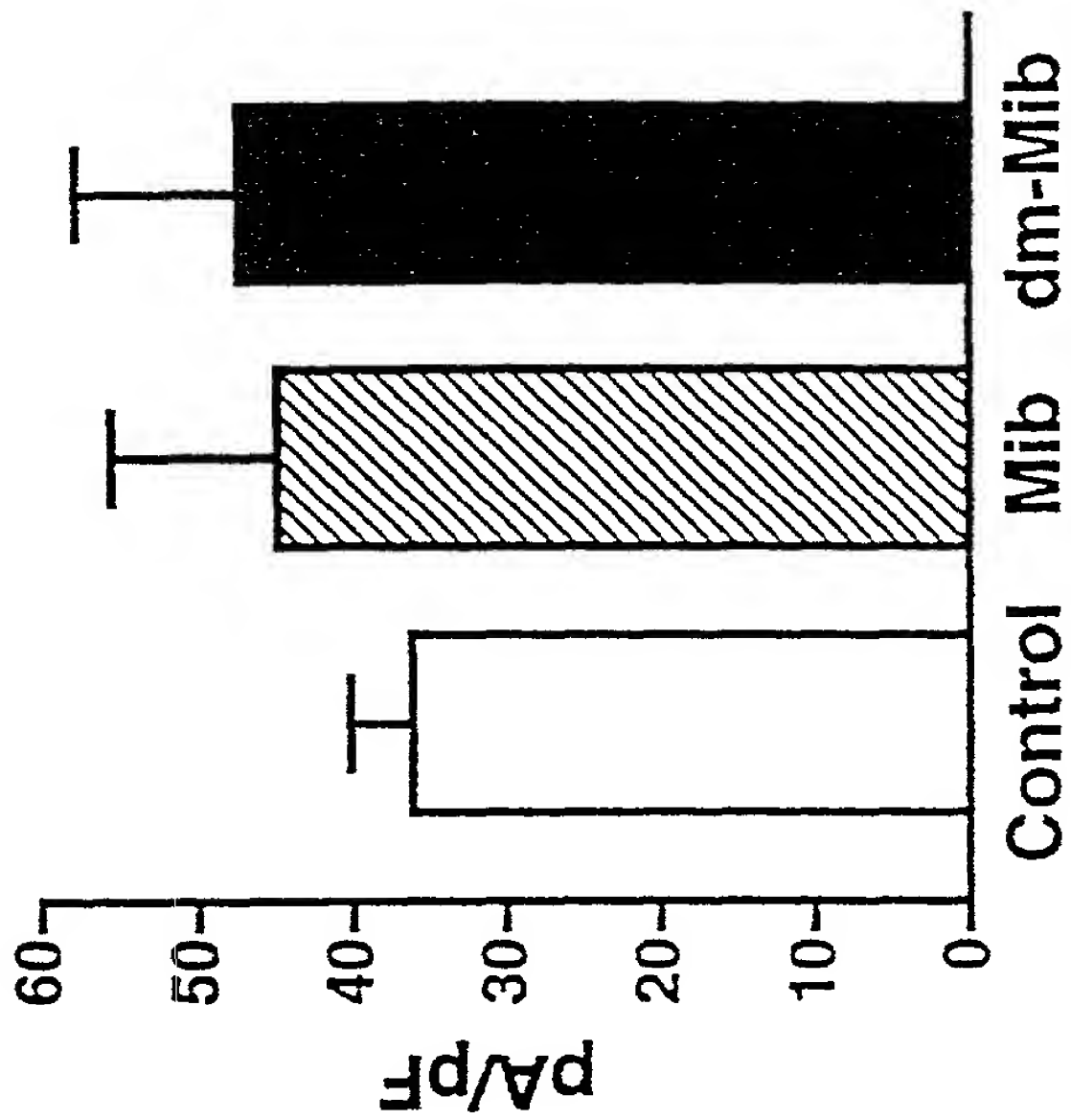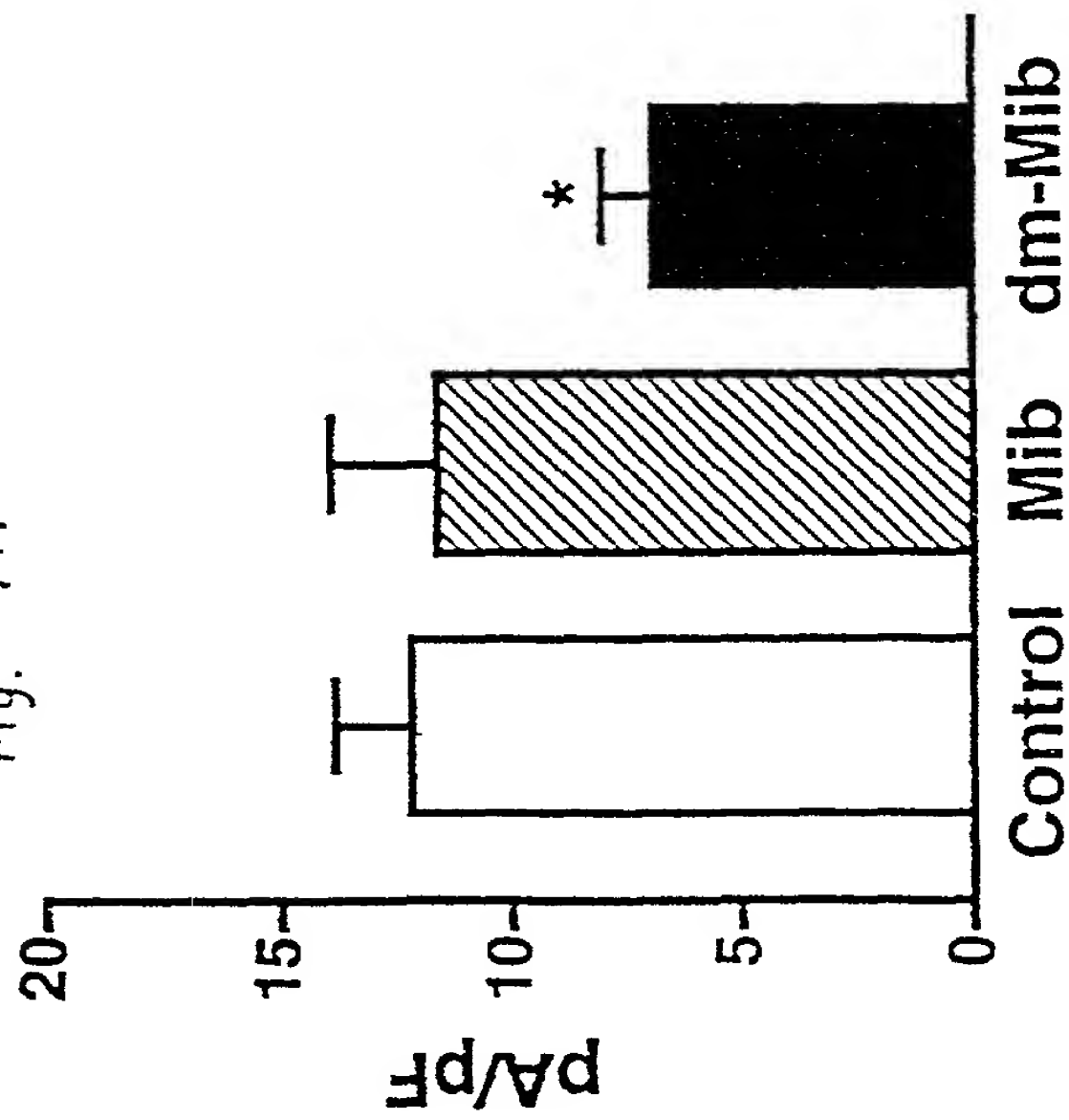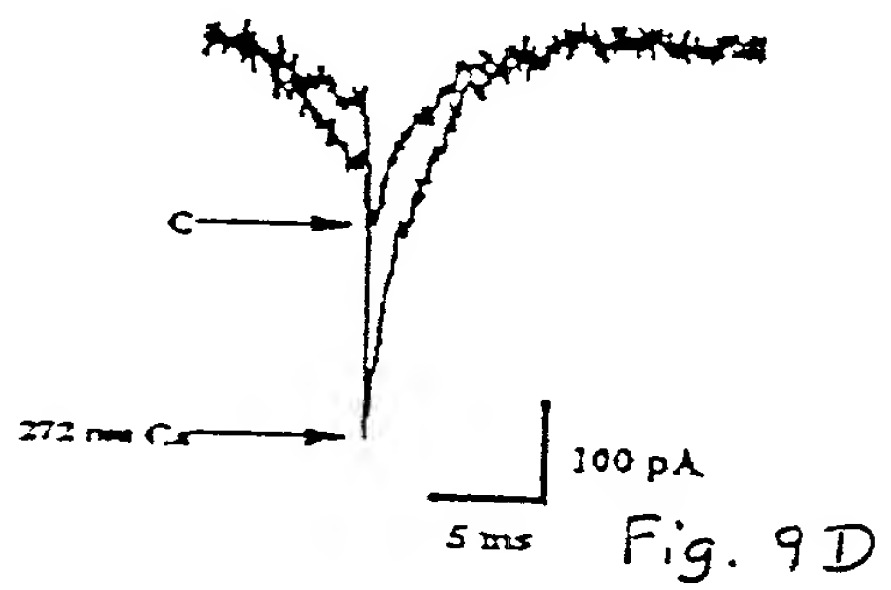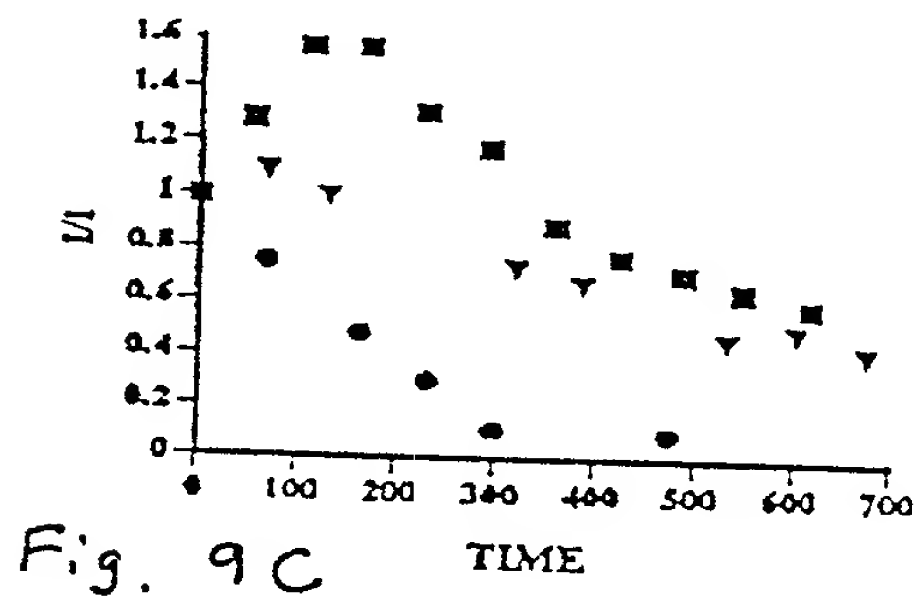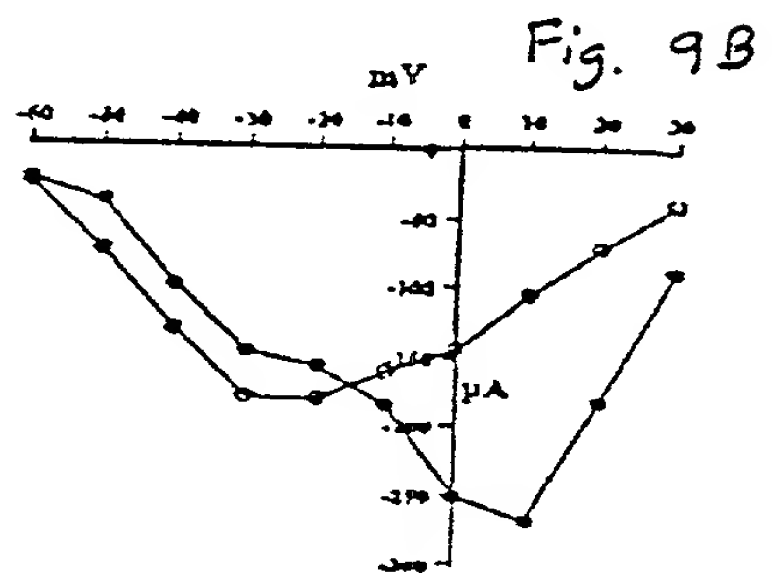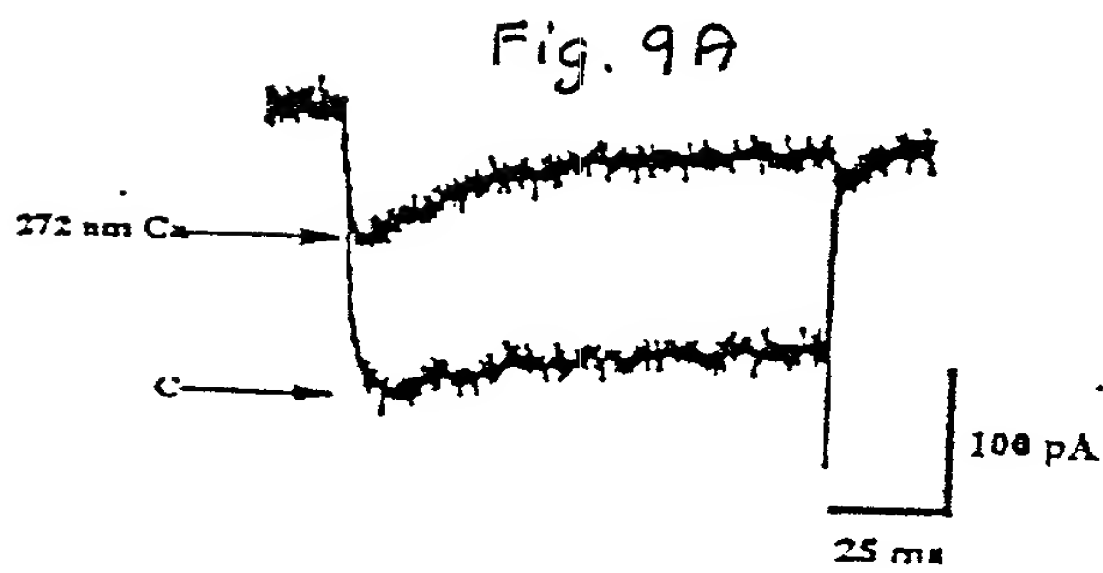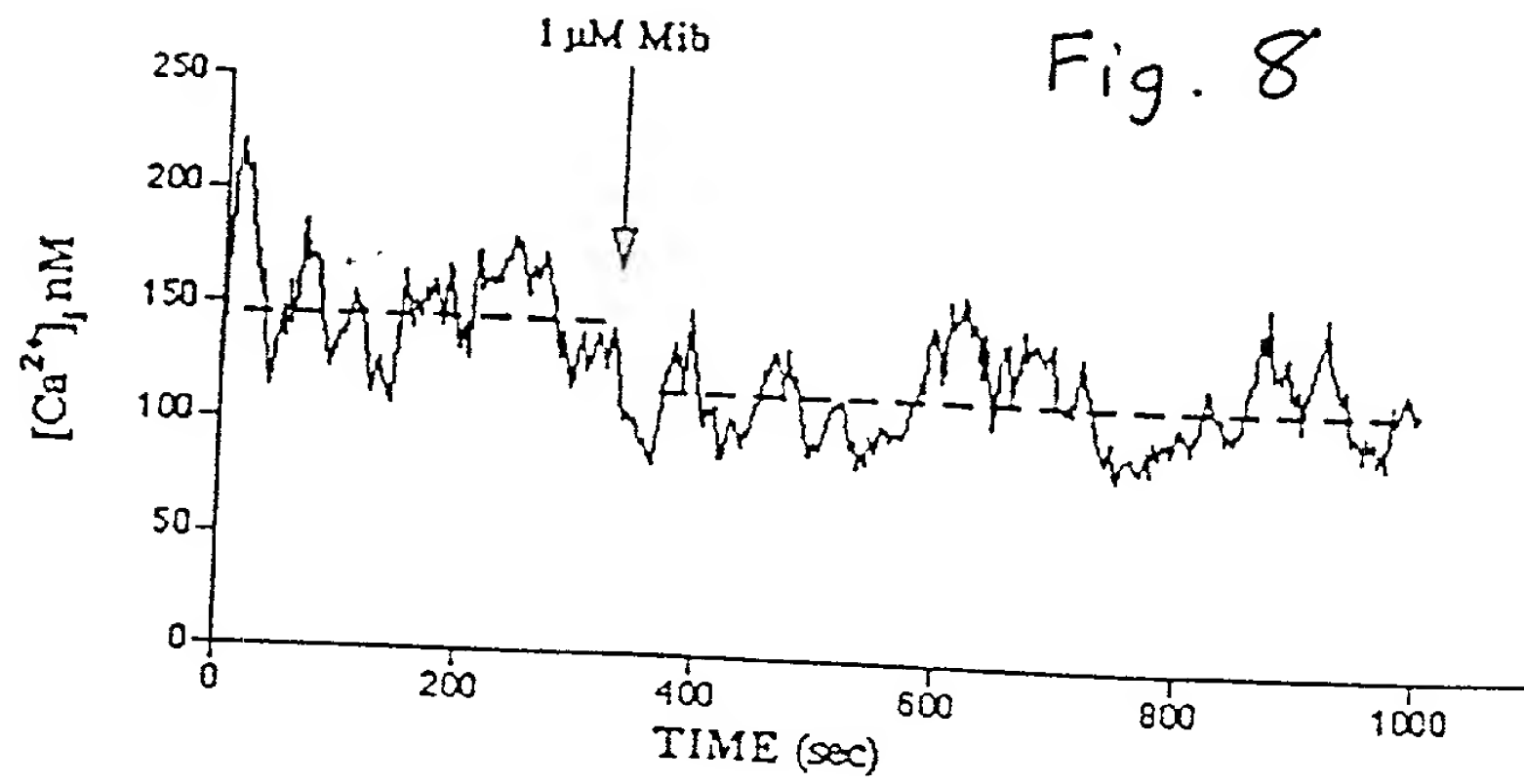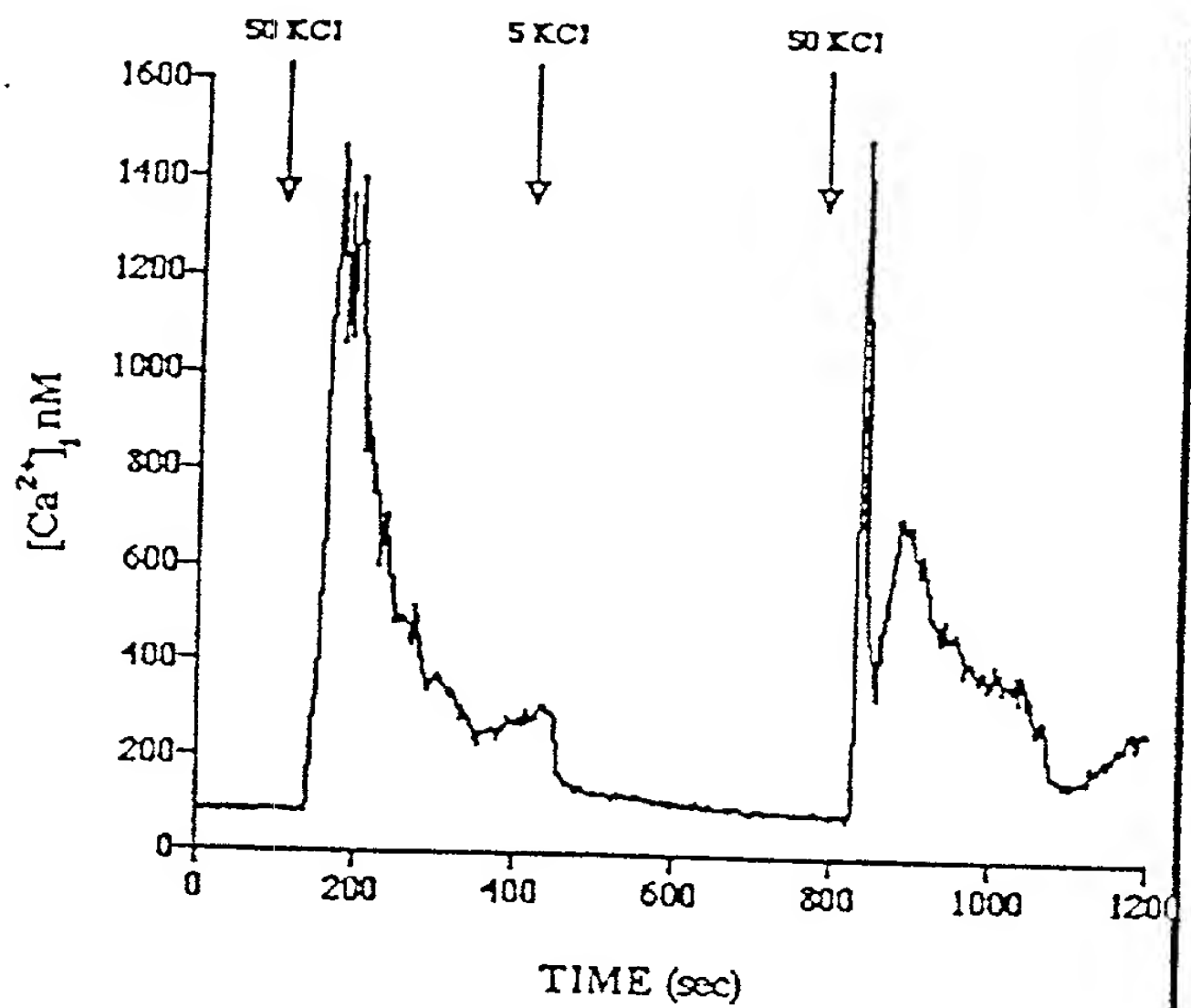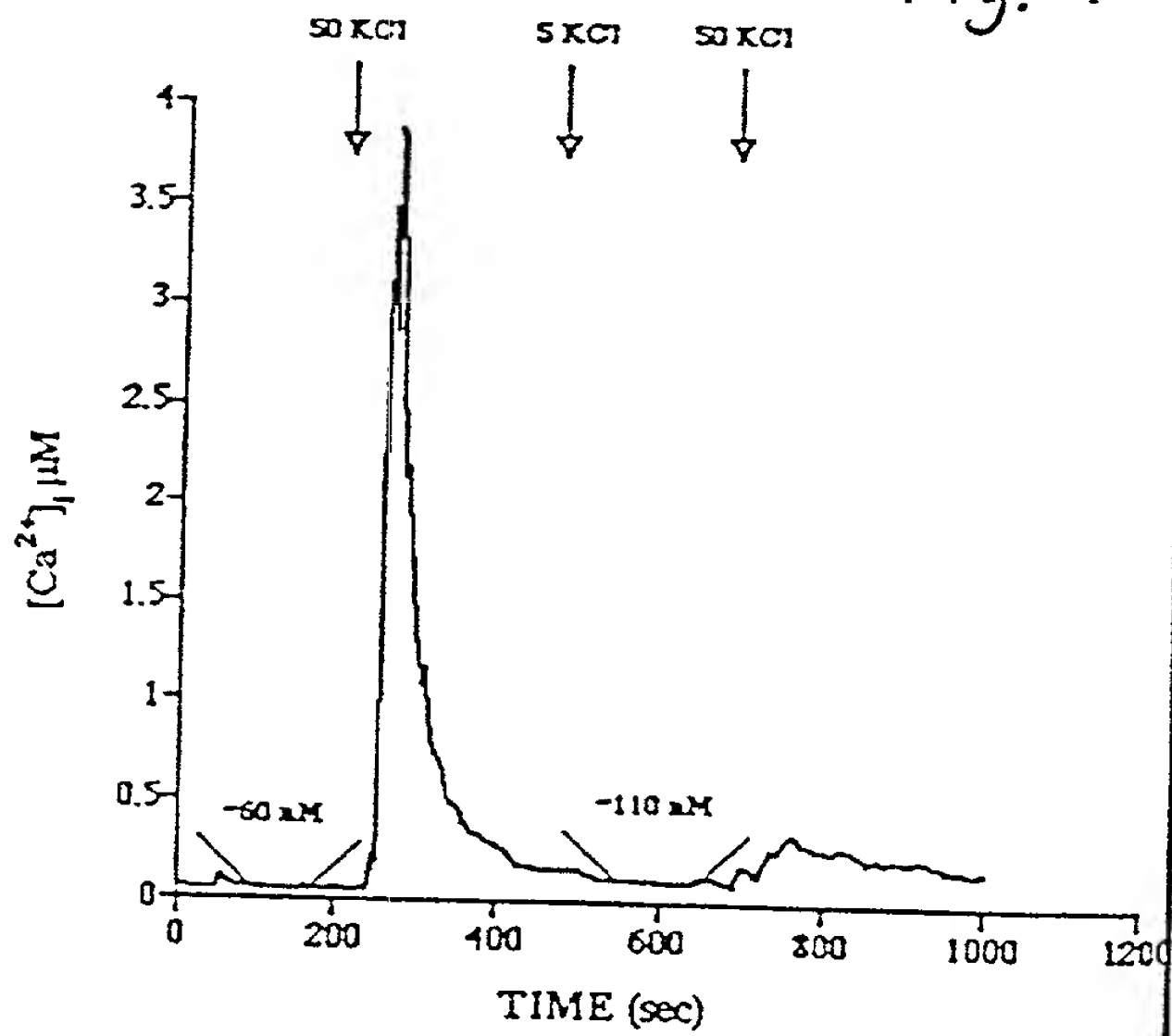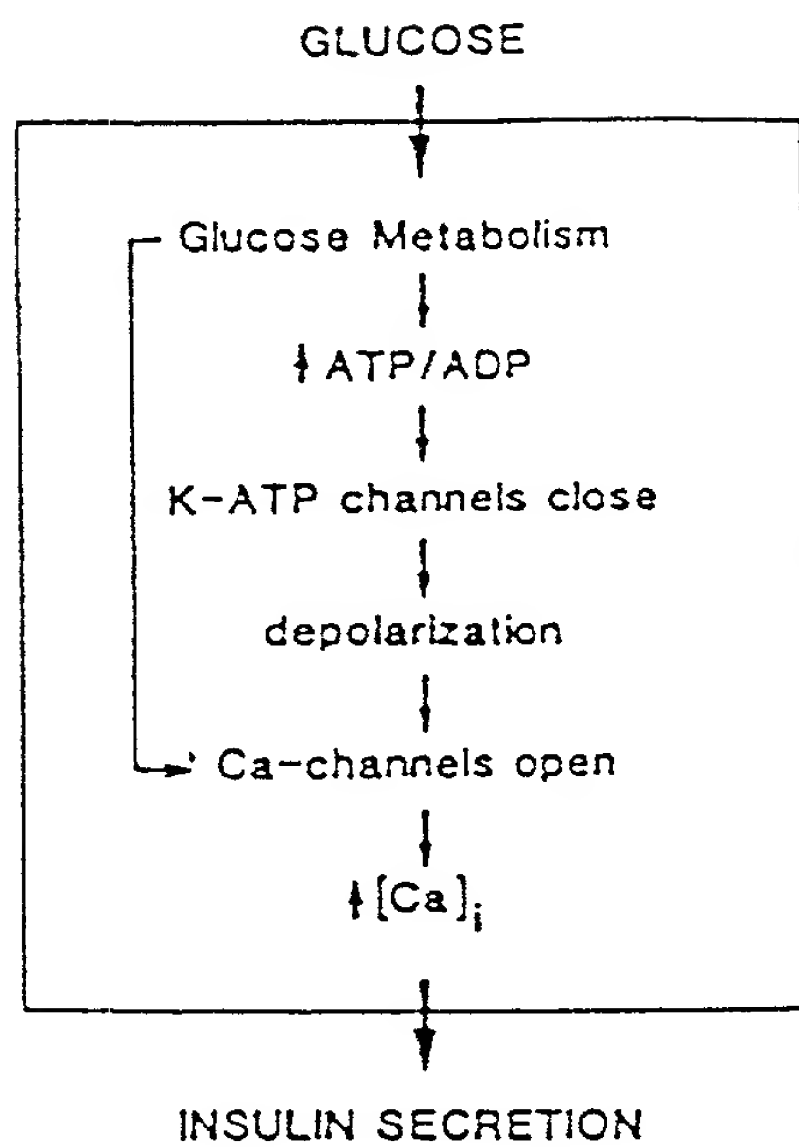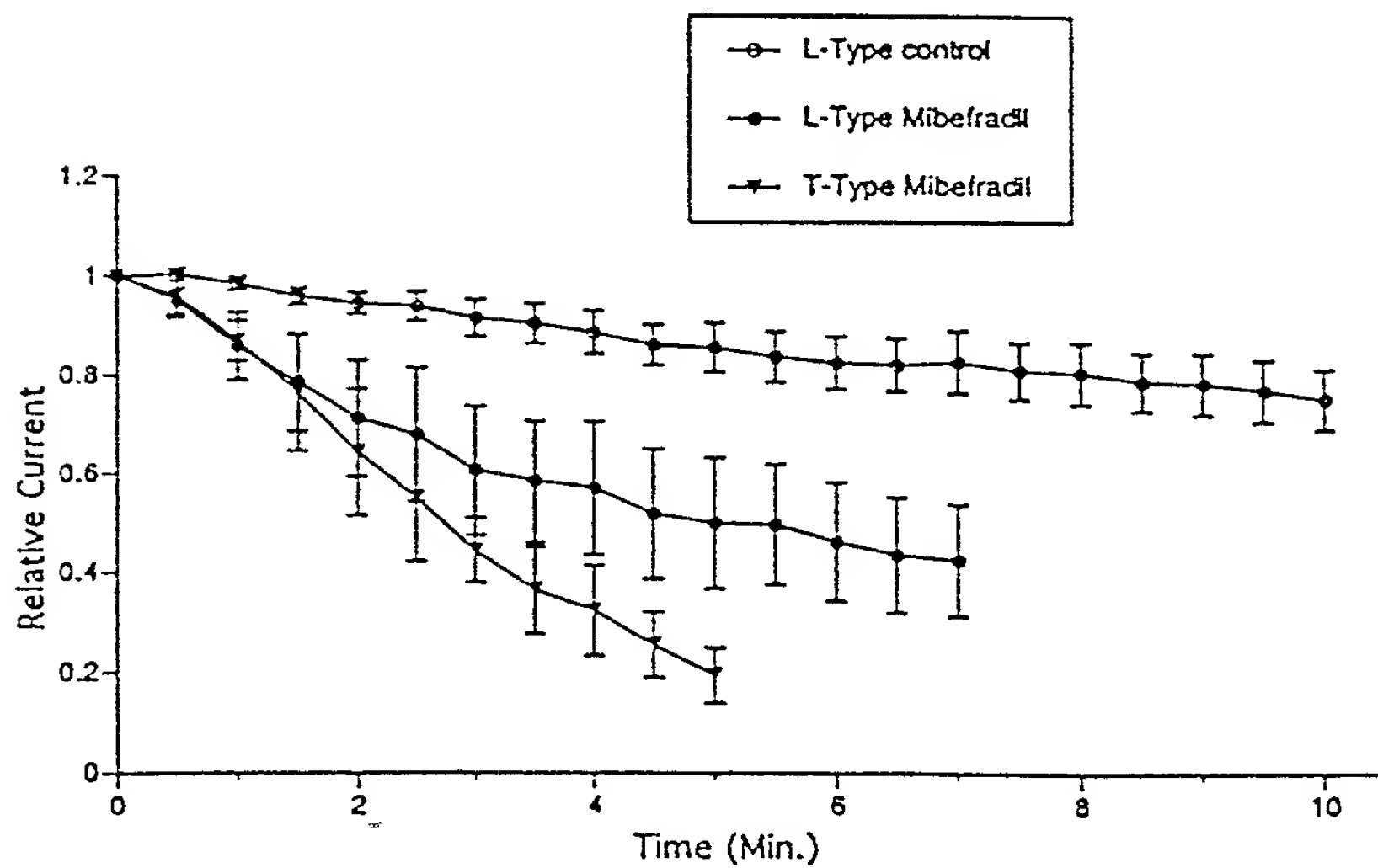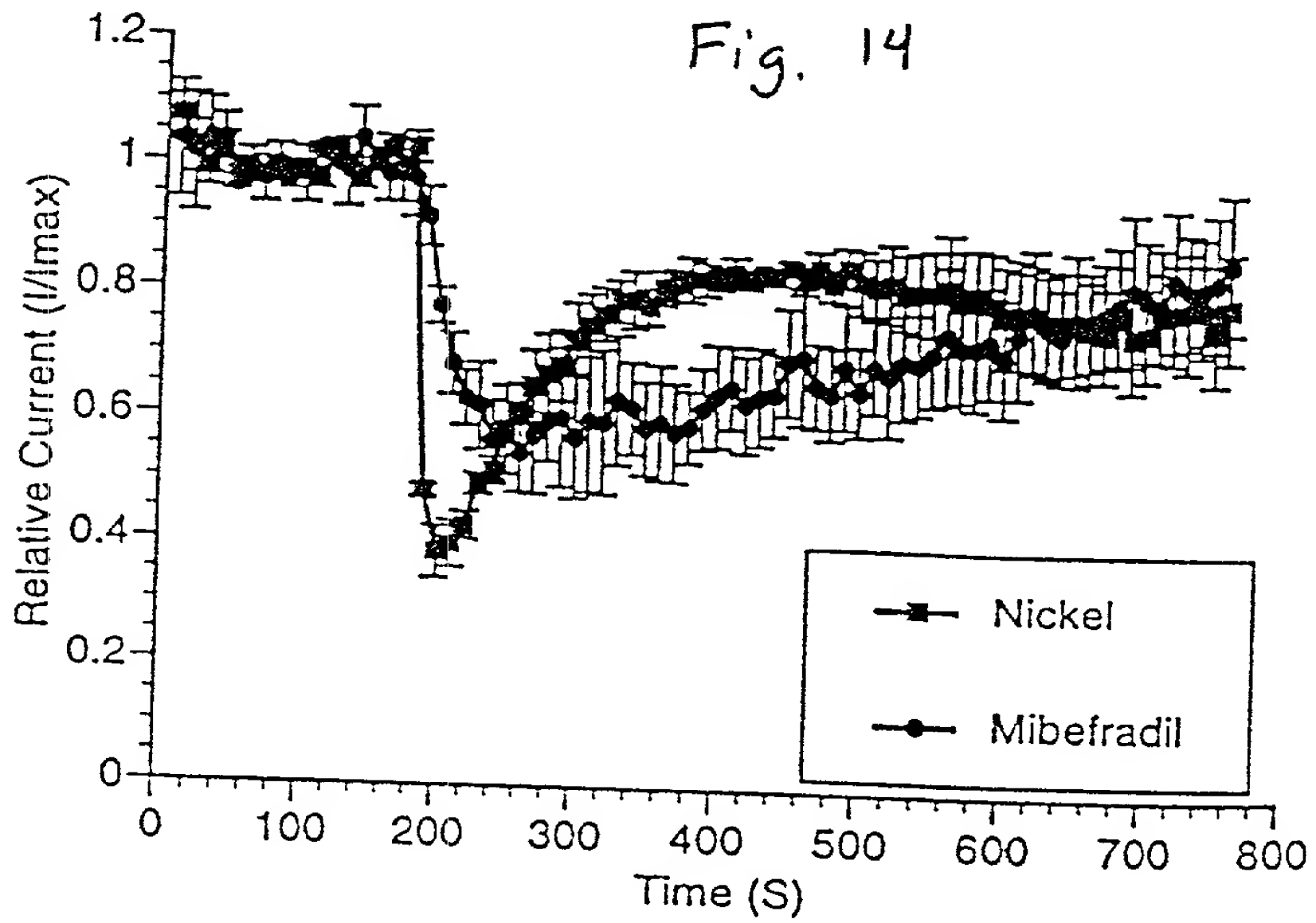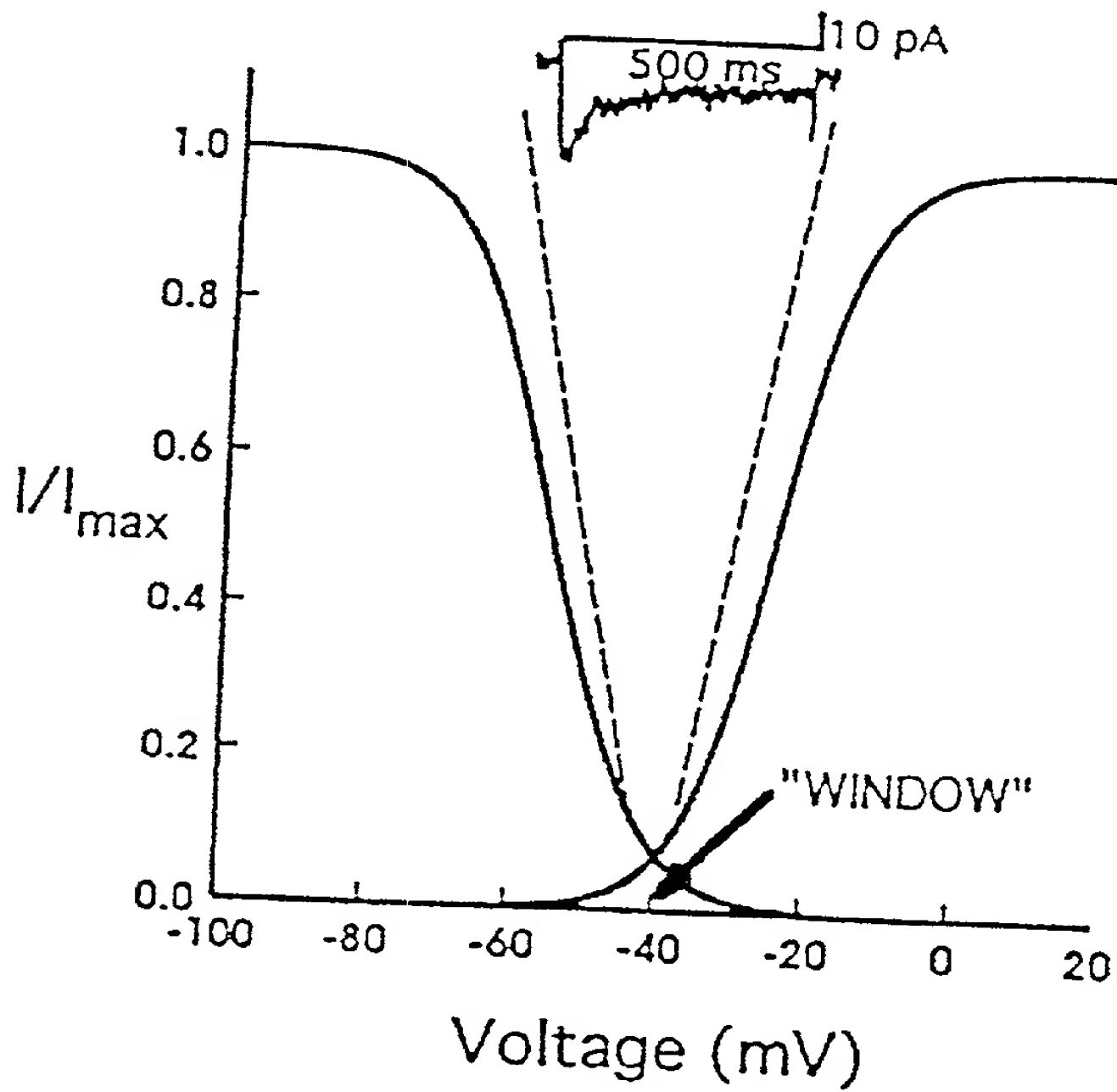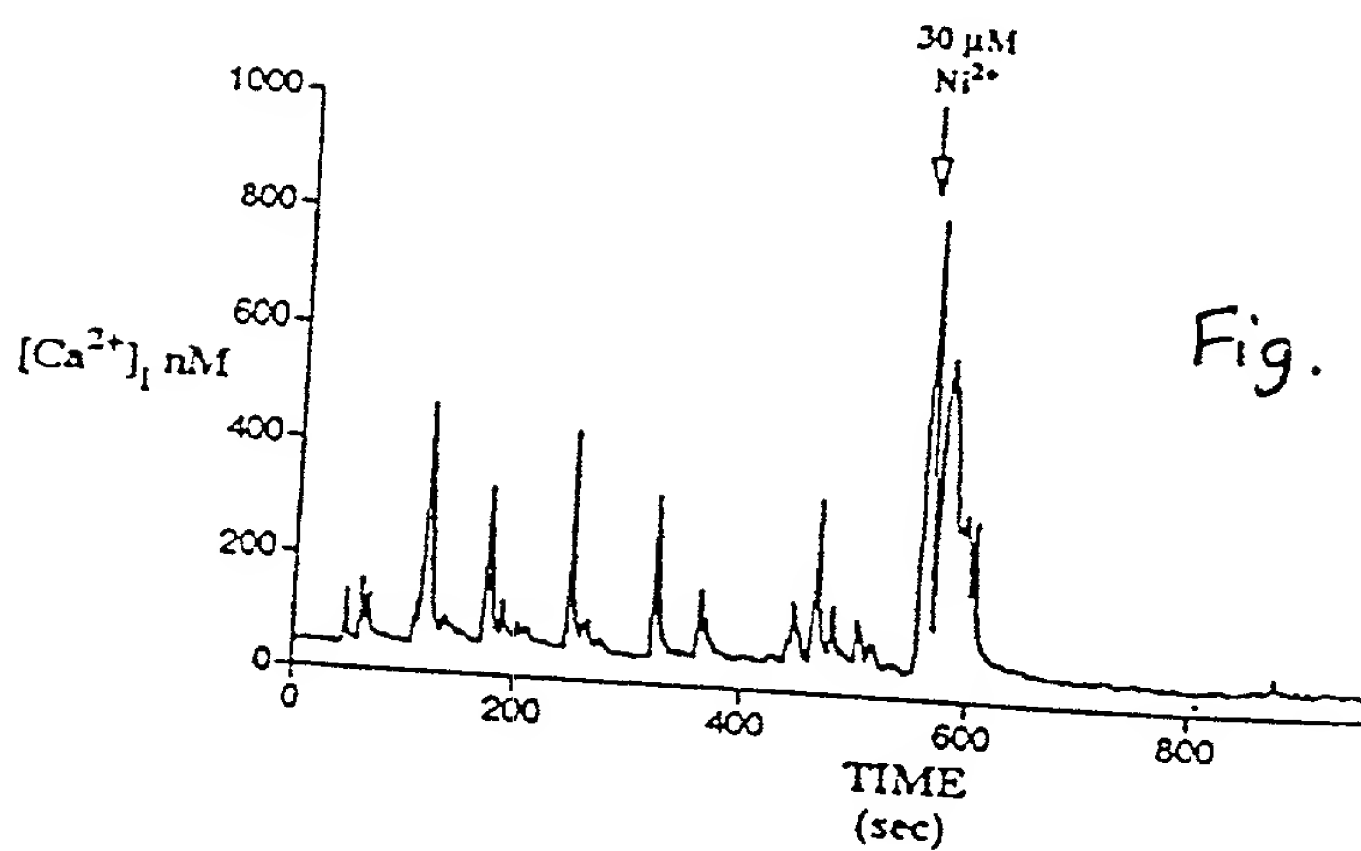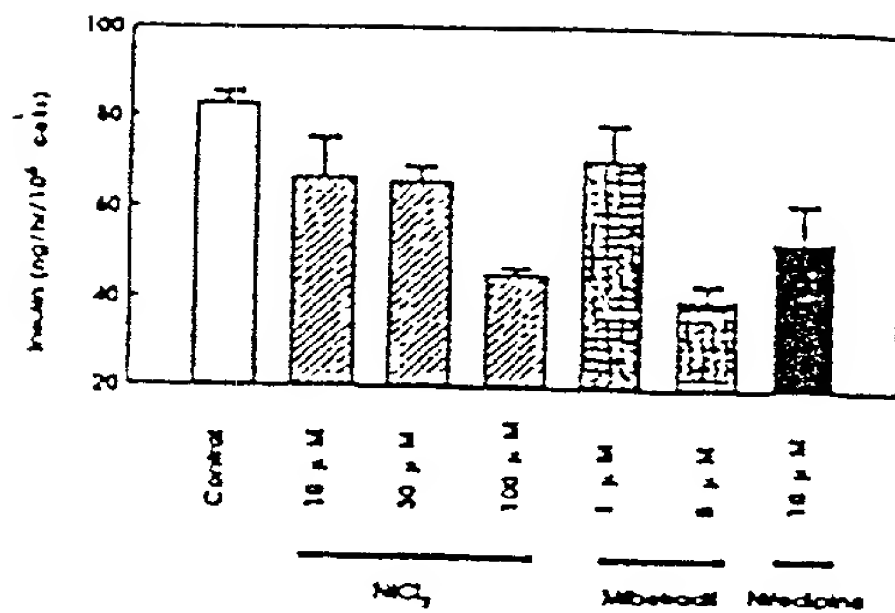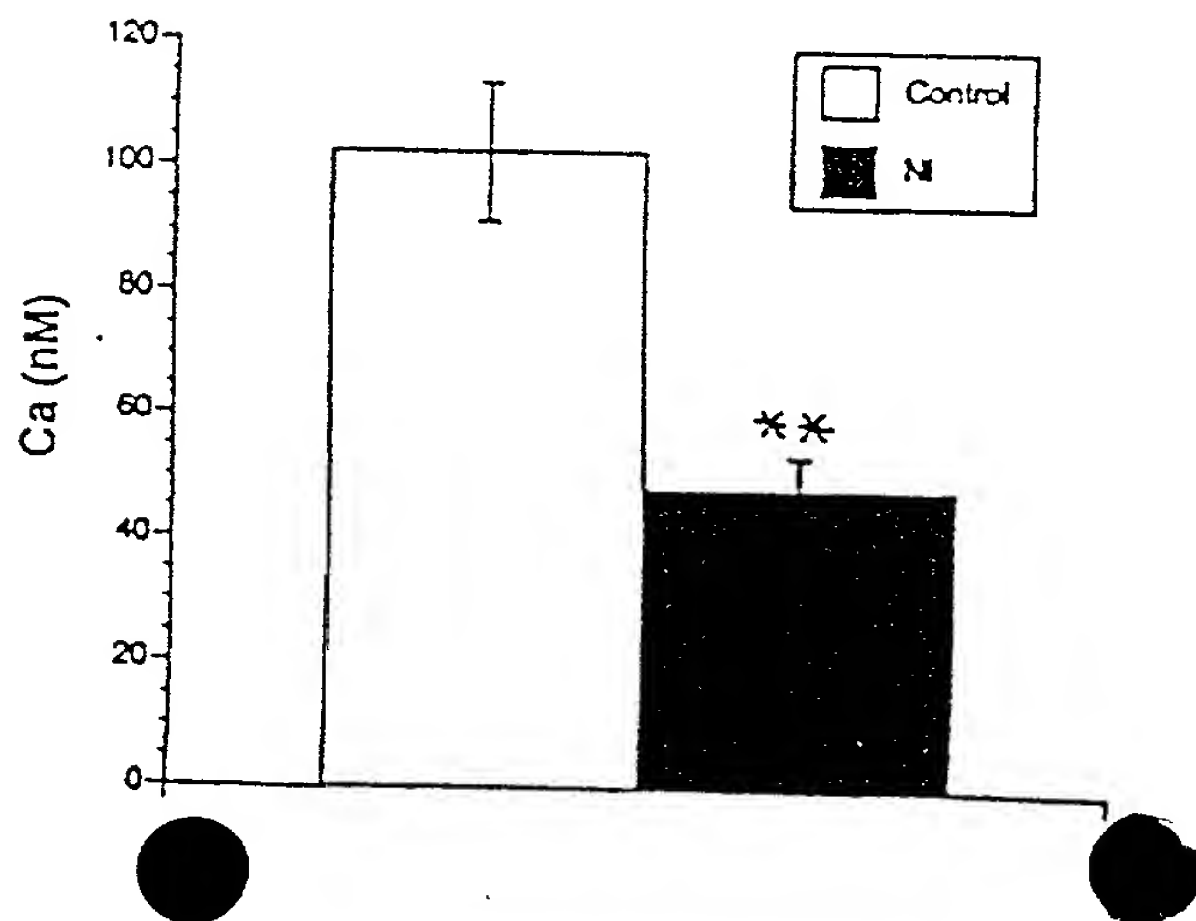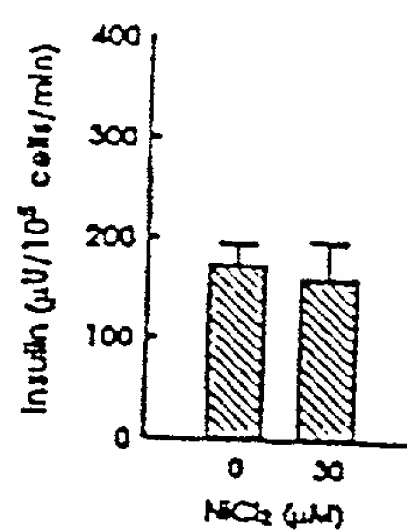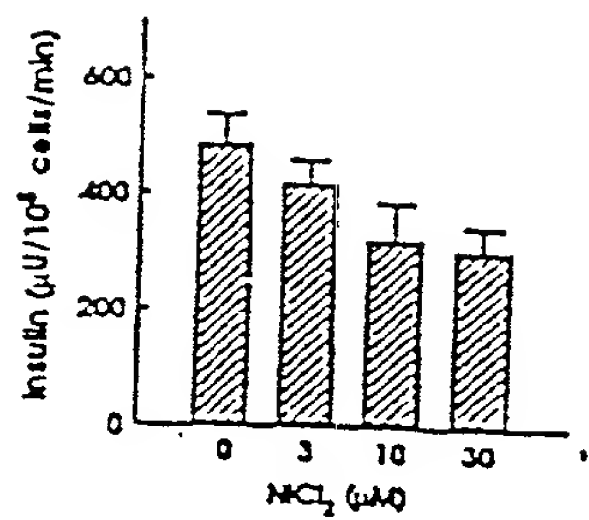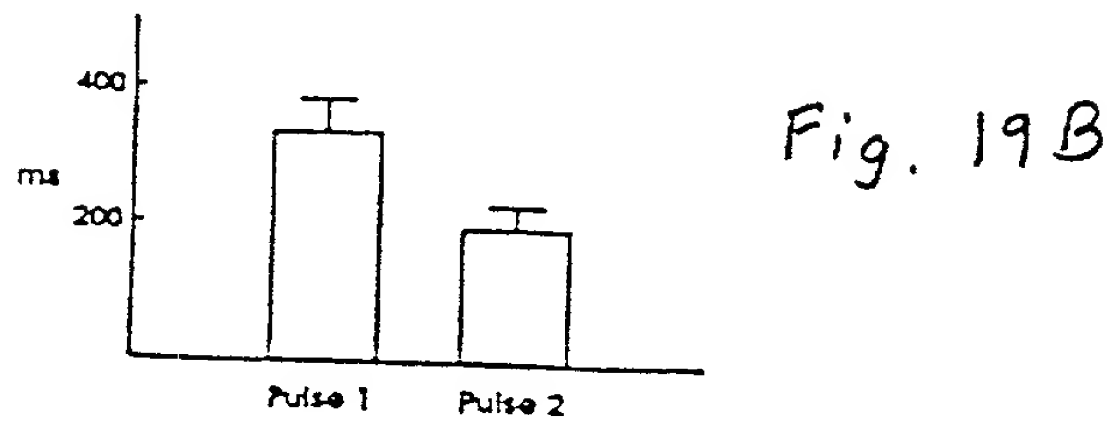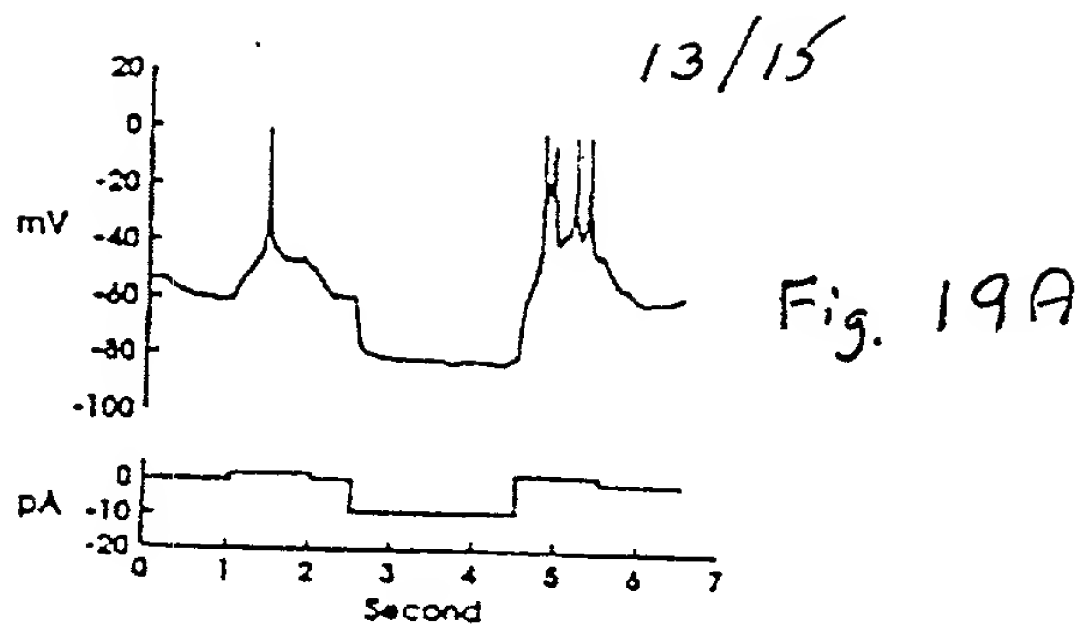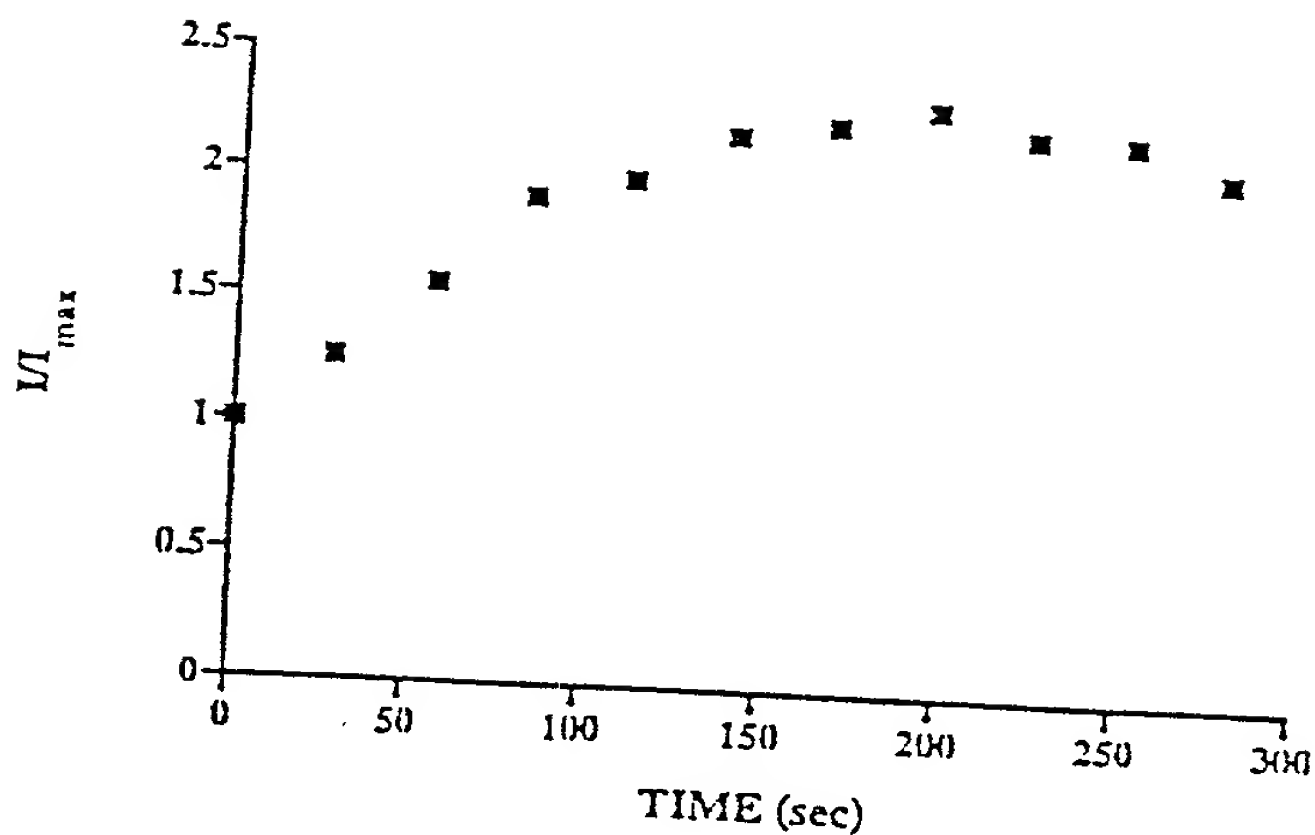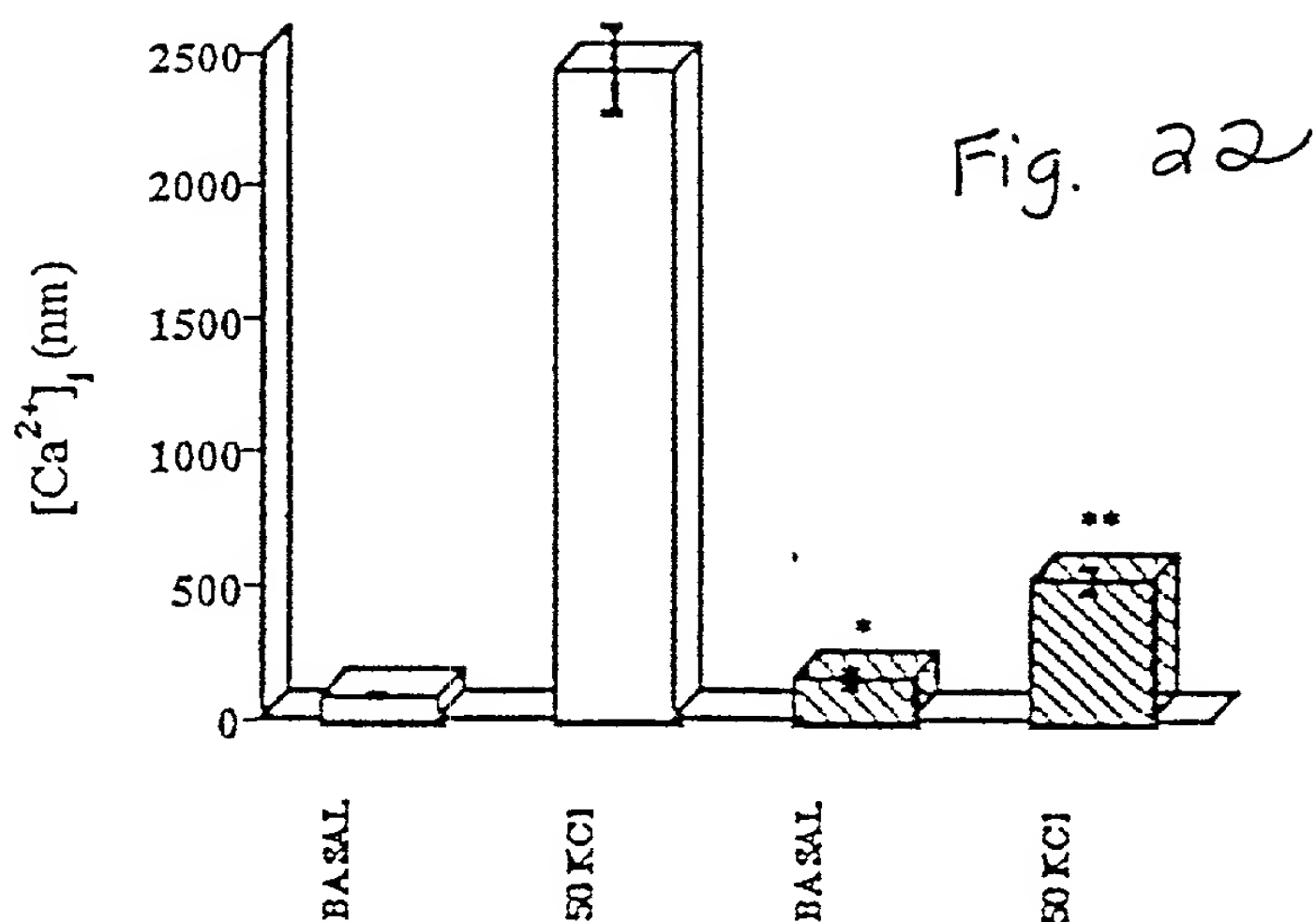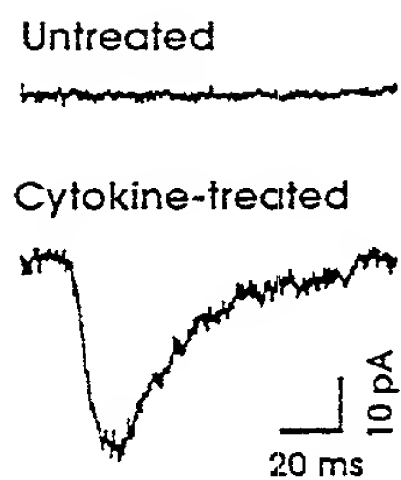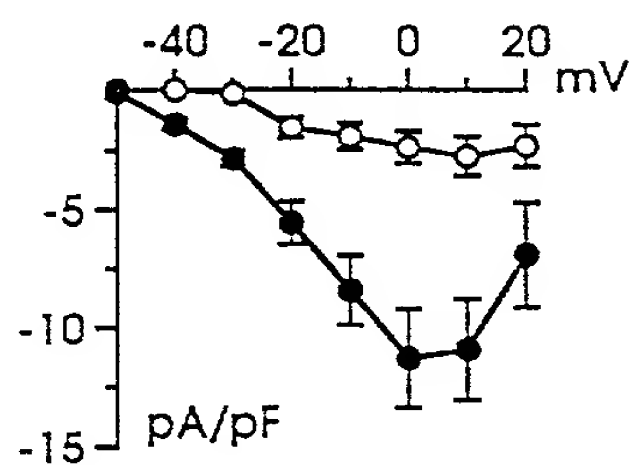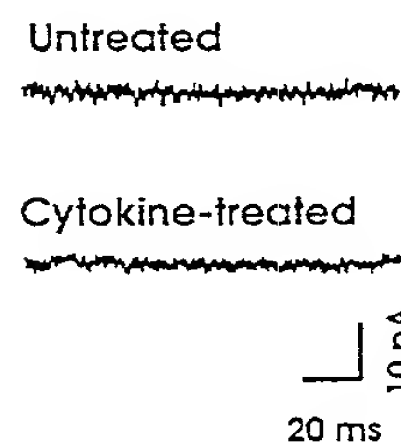
Fig. 23D.

Fig. 23E.

Fig. 23F.

10 μM NiCl$_2$

Control

5 pA

20 ms
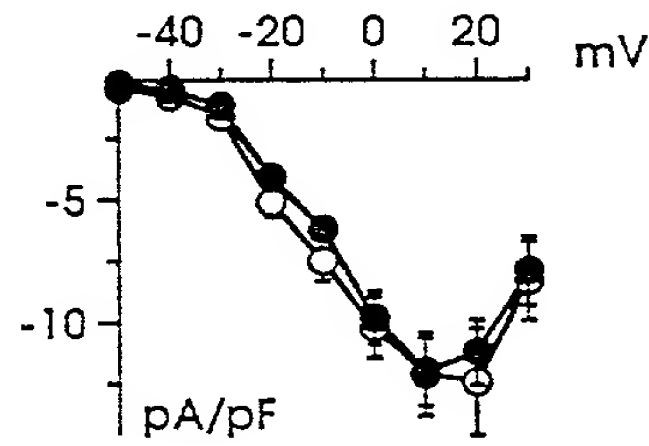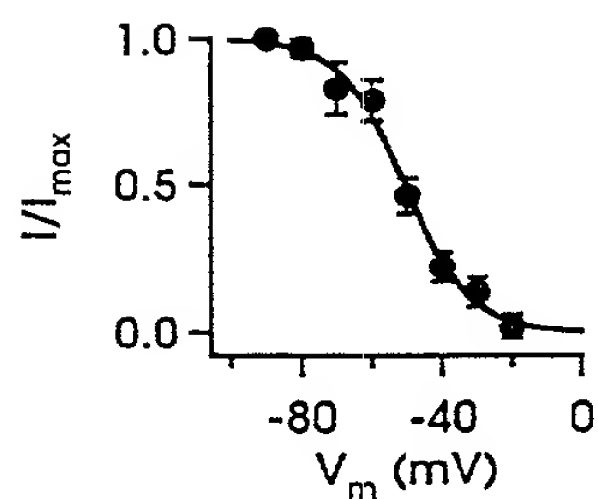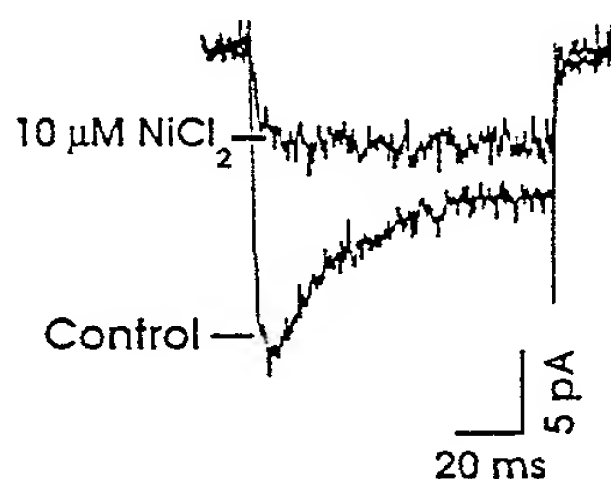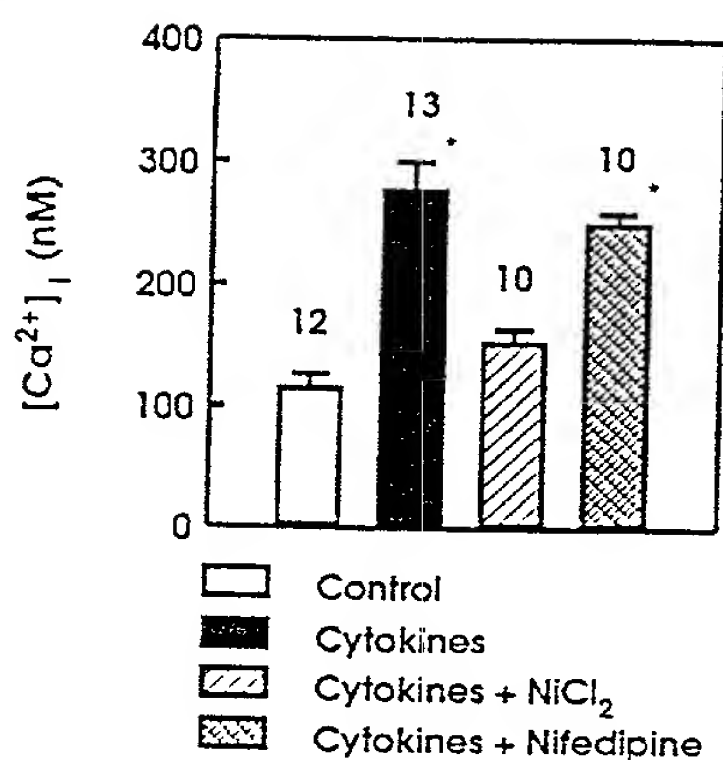
Fig. 24 A.

Fig. 24 B.
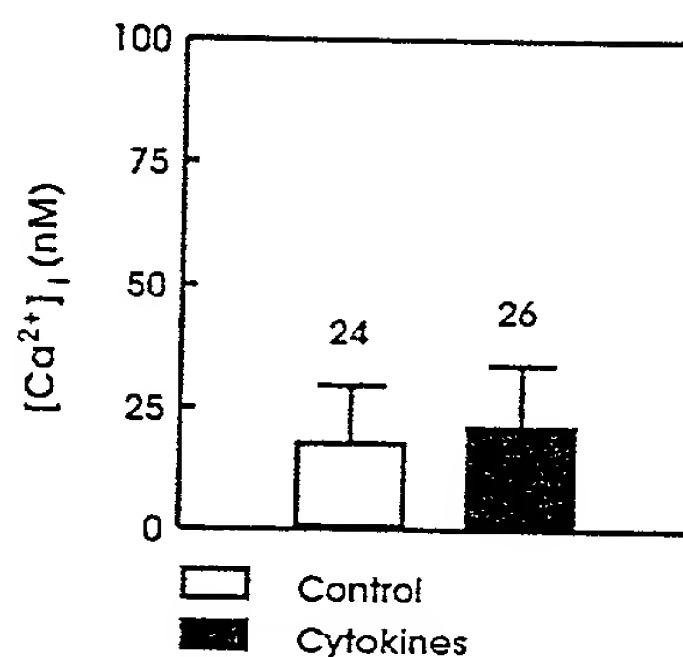
Fig. 25 A.
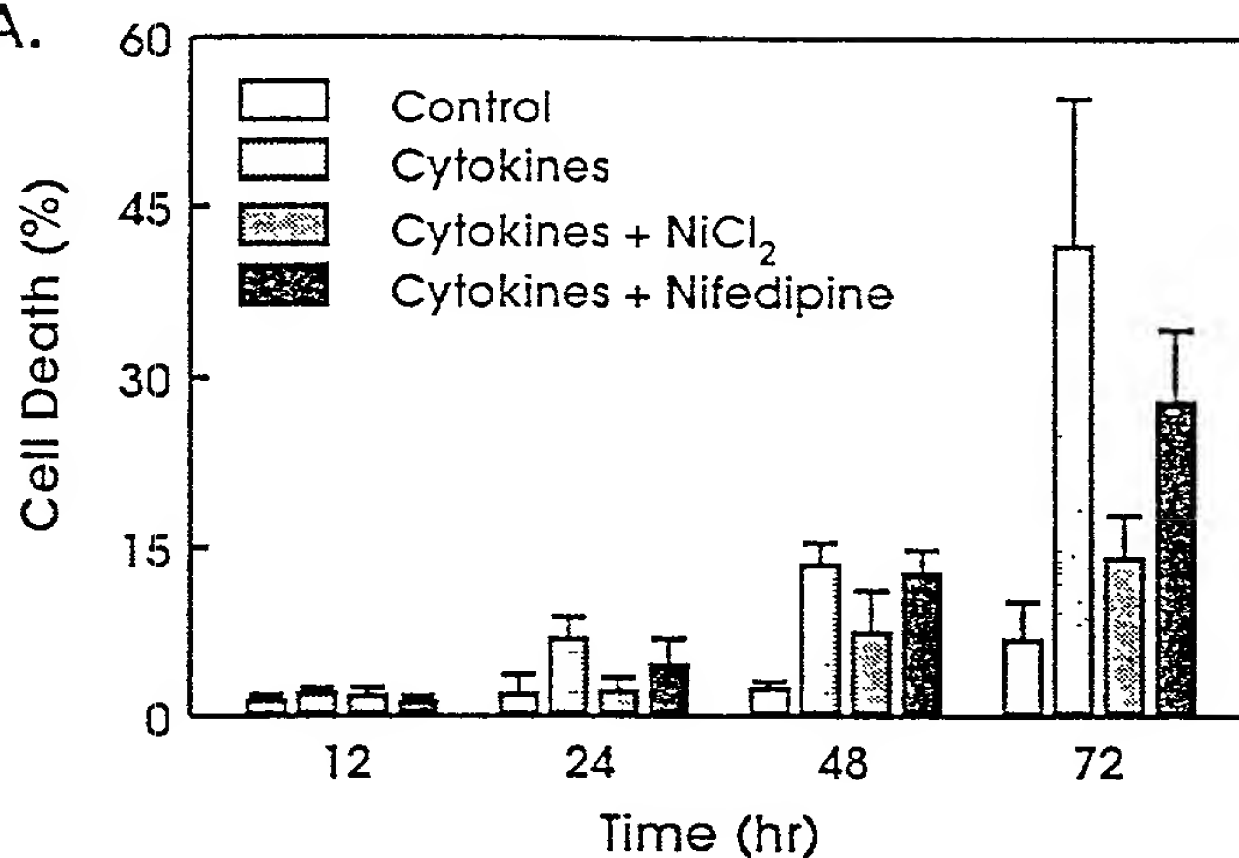
Fig. 25 B.
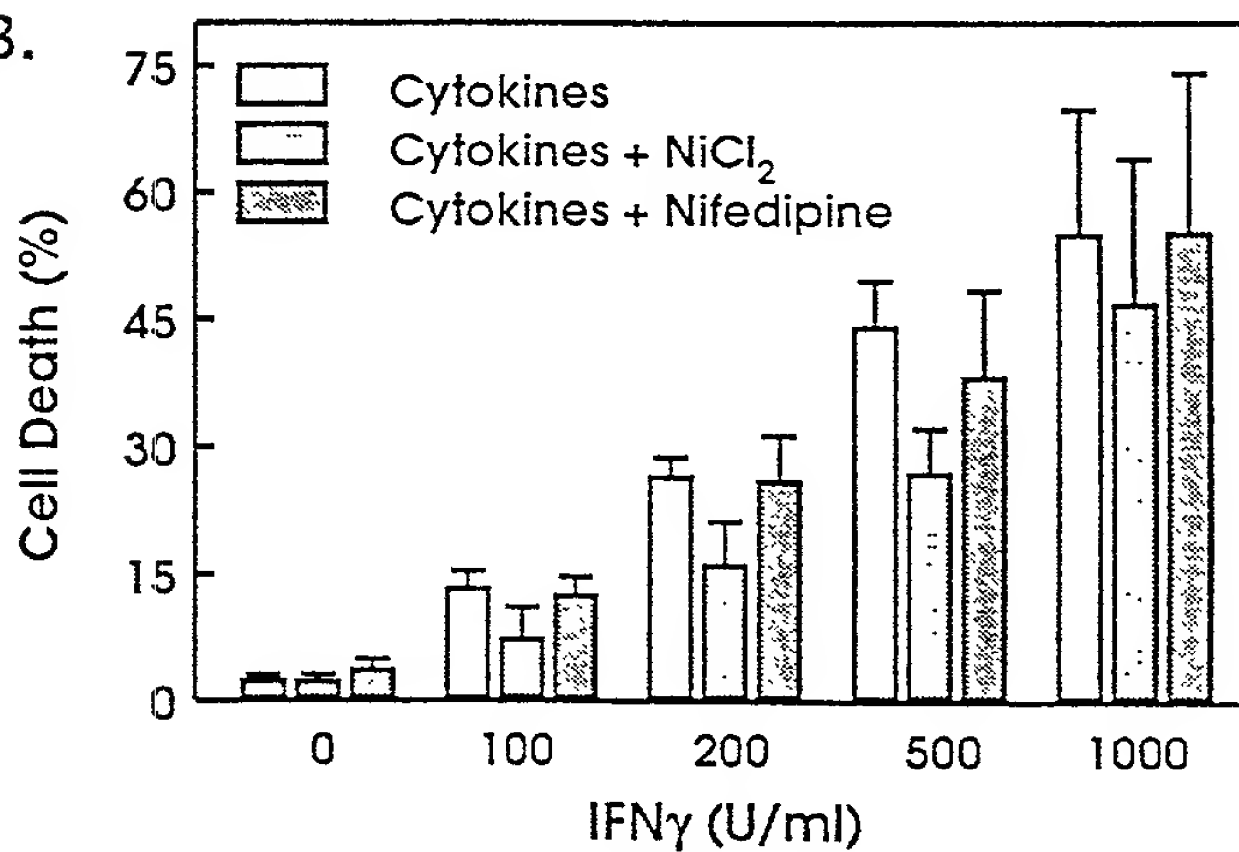
SEQUENCE LISTING

DNASIS· DNA Translation [T-INS]
File Name : T-INS
Range :   2 - 7286   Mode : Normal
Codon Table : Universal

start of SEQ 3 + 4

```
               10              19              28              37              46              55
5'  GAG CTG AGC TGA ACT GGC CCT CCT GGG GAC TCA GCA AGC TCT CTA GAG CCC CCC
    --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- ---
     E   L   S   *   T   G   P   P   G   D   S   A   S   S   L   E   P   P

                       64              73              82              91             100             109
    ACA TGC TCC CCC ACC GGG GTC CCC CGG TTG CGT GAG GAC ACC TCC TCT GAG GGG
    --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- ---
     T   C   S   P   T   G   V   P   R   L   R   E   D   T   S   S   E   G
```

start of SEQ ID NO:1 + SEQ ID NO:2 (coding regions)

```
           118             127             136             145             154             163
    CTC CGC TCG CCC CTC TTC GGA CCC CCC GGG GCC CCG GCT GGC CAG AGG ATG GAC
    --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- ---
     L   R   S   P   L   F   G   P   P   G   A   P   A   G   Q   R   M   D

           172             181             190             199             208             217
    GAG GAG GAG GAT GGA GCG GGC GCC GAG GAG TCG GGA CAG CCC CGT AGC TTC ACG
    --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- ---
     E   E   E   D   G   A   G   A   E   E   S   G   Q   P   R   S   F   T

           226             235             244             253             262             271
    CAG CTC AAC GAC CTG TCC GGG GCC GGG GGC CGG CAG GGG CCG GGG TCG ACG GAA
    --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- ---
     Q   L   N   D   L   S   G   A   G   G   R   Q   G   P   G   S   T   E

           280             289             298             307             316             325
    AAG GAC CCG GGC AGC GCG GAC TCC GAG GCG GAG GGG CTG CCG TAC CCG GCG CTA
    --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- ---
     K   D   P   G   S   A   D   S   E   A   E   G   L   P   Y   P   A   L

           334             343             352             361             370             379
    GCC CCG GTG GTT TTC TTC TAC TTG AGC CAG GAC AGC CGC CCG CGG AGC TGG TGT
    --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- ---
     A   P   V   V   F   F   Y   L   S   Q   D   S   R   P   R   S   W   C

           388             397             406             415             424             433
    CTC CGC ACG GTC TGT AAC CCG TGG TTC GAG CGA GTC AGT ATG CTG GTC ATT CTT
    --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- ---
     L   R   T   V   C   N   P   W   F   E   R   V   S   M   L   V   I   L

           442             451             460             469             478             487
    CTC AAC TGT GTG ACT CTG GGT ATG TTC AGG CCG TGT GAG GAC ATT GCC TGT GAC
    --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- ---
     L   N   C   V   T   L   G   M   F   R   P   C   E   D   I   A   C   D

           496             505             514             523             532             541
    TCC CAG CGC TGC CGG ATC CTG CAG GCC TTC GAT GAC TTC ATC TTT GCC TTC TTT
    --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- ---
     S   Q   R   C   R   I   L   Q   A   F   D   D   F   I   F   A   F   F

           550             559             568             577             586             595
    GCT GTG GAA ATG GTG GTG AAG ATG GTG GCC TTG GGC ATC TTT GGG AAG AAA TGT
    --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- ---
     A   V   E   M   V   V   K   M   V   A   L   G   I   F   G   K   K   C

           604             613             622             631             640             649
    TAC CTG GGA GAC ACT TGG AAC CGG CTT GAC TTT TTC ATT GTC ATT GCA GGG ATG
    --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- ---
     Y   L   G   D   T   W   N   R   L   D   F   F   I   V   I   A   G   M
```

DNASIS DNA Translation [T-INS]

```
        658            667            676            685            694            703
CTG GAG TAT TCG CTG GAC CTG CAG AAC GTC AGC TTC TCC GCA GTC AGG ACA GTC
--- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- ---
 L   E   Y   S   L   D   L   Q   N   V   S   F   S   A   V   R   T   V

        712            721            730            739            748            757
CGT GTG CTG CGA CCG CTC AGG GCC ATT AAC CGG GTG CCC AGC ATG CGC ATT CTC
--- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- ---
 R   V   L   R   P   L   R   A   I   N   R   V   P   S   M   R   I   L

        766            775            784            793            802            811
GTC ACA TTA CTG CTG GAC ACC TTG CCT ATG CTG GGC AAC GTC CTG CTG CTC TGT
--- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- ---
 V   T   L   L   L   D   T   L   P   M   L   G   N   V   L   L   L   C

        820            829            838            847            856            865
TTC TTC GTC TTT TTC ATC TTT GGC ATC GTG GGC GTC CAG CTG TGG GCA GGA CTG
--- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- ---
 F   F   V   F   F   I   F   G   I   V   G   V   Q   L   W   A   G   L

        874            883            892            901            910            919
CTT CGC AAC CGA TGC TTC CTC CCC GAG AAC TTC AGC CTC CCC CTG AGC GTG GAC
--- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- ---
 L   R   N   R   C   F   L   P   E   N   F   S   L   P   L   S   V   D

        928            937            946            955            964            973
CTG GAG CCT TAT TAC CAG ACA GAG AAT GAG GAC GAG AGC CCC TTC ATC TGC TCT
--- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- ---
 L   E   P   Y   Y   Q   T   E   N   E   D   E   S   P   F   I   C   S

        982            991           1000           1009           1018           1027
CAG CCT CGG GAG AAT GGC ATG AGA TCC TGC AGG AGT GTG CCC ACA CTG CGT GGG
--- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- ---
 Q   P   R   E   N   G   M   R   S   C   R   S   V   P   T   L   R   G

       1036           1045           1054           1063           1072           1081
GAA GGC GGT GGT GGC CCA CCC TGC AGT CTG GAC TAT GAG ACC TAT AAC AGT TCC
--- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- ---
 E   G   G   G   G   P   P   C   S   L   D   Y   E   T   Y   N   S   S

       1090           1099           1108           1117           1126           1135
AGC AAC ACC ACC TGT GTC AAC TGG AAC CAG TAC TAT ACC AAC TGC TCT GCG GGC
--- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- ---
 S   N   T   T   C   V   N   W   N   Q   Y   Y   T   N   C   S   A   G

       1144           1153           1162           1171           1180           1189
GAG CAC AAC CCC TTC AAA GGC GCC ATC AAC TTT GAC AAC ATT GGC TAT GCC TGG
--- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- ---
 E   H   N   P   F   K   G   A   I   N   F   D   N   I   G   Y   A   W

       1198           1207           1216           1225           1234           1243
ATC GCC ATC TTC CAG GTC ATC ACA CTG GAG GGC TGG GTC GAC ATC ATG TAC TTC
--- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- ---
 I   A   I   F   Q   V   I   T   L   E   G   W   V   D   I   M   Y   F

       1252           1261           1270           1279           1288           1297
GTA ATG GAC GCT CAC TCC TTC TAC AAC TTC ATC TAC TTC ATT CTT CTC ATC ATC
--- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- ---
 V   M   D   A   H   S   F   Y   N   F   I   Y   F   I   L   L   I   I

       1306           1315           1324           1333           1342           1351
GTG GGC TCC TTC TTC ATG ATC AAC CTG TGC CTG GTG GTG ATT GCC ACG CAG TTC
--- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- ---
 V   G   S   F   F   M   I   N   L   C   L   V   V   I   A   T   Q   F
```

DNASIS   DNA   Translation [T-INS]

```
         1360        1369        1378        1387        1396        1405
TCC GAG ACC AAA CAG CGG GAG AGT CAG CTG ATG CGG GAG CAG CGT GTA CGA TTC
--- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- ---
 S   E   T   K   Q   R   E   S   Q   L   M   R   E   Q   R   V   R   F

         1414        1423        1432        1441        1450        1459
CTG TCC AAT GCT AGC ACC CTG GCA AGC TTC TCT GAG CCA GGC AGC TGC TAT GAG
--- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- ---
 L   S   N   A   S   T   L   A   S   F   S   E   P   G   S   C   Y   E

         1468        1477        1486        1495        1504        1513
GAG CTA CTC AAG TAC CTG GTG TAC ATC CTC CGA AAA GCA GCC CGA AGG CTG GCC
--- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- ---
 E   L   L   K   Y   L   V   Y   I   L   R   K   A   A   R   R   L   A

         1522        1531        1540        1549        1558        1567
CAG GTC TCT AGG GCT ATA GGC GTG CGG GCT GGG CTG CTC AGC AGC CCA GTG GCC
--- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- ---
 Q   V   S   R   A   I   G   V   R   A   G   L   L   S   S   P   V   A

         1576        1585        1594        1603        1612        1621
CGT AGT GGG CAG GAG CCC CAG CCC AGT GGC AGC TGC ACT CGC TCA CAC CGT CGT
--- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- ---
 R   S   G   Q   E   P   Q   P   S   G   S   C   T   R   S   H   R   R

         1630        1639        1648        1657        1666        1675
CTG TCT GTC CAC CAC CTG GTC CAC CAC CAT CAC CAC CAC CAT CAC CAC TAC CAC
--- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- ---
 L   S   V   H   H   L   V   H   H   H   H   H   H   H   H   H   Y   H

         1684        1693        1702        1711        1720        1729
CTG GGT AAT GGG ACG CTC AGA GTT CCC CGG GCC AGC CCA GAG ATC CAG GAC AGG
--- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- ---
 L   G   N   G   T   L   R   V   P   R   A   S   P   E   I   Q   D   R

         1738        1747        1756        1765        1774        1783
GAT GCC AAT GGG TCT CGC CGG CTC ATG CTA CCA CCA CCC TCT ACA CCC ACT CCC
--- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- ---
 D   A   N   G   S   R   R   L   M   L   P   P   P   S   T   P   T   P

         1792        1801        1810        1819        1828        1837
TCT GGG GGC CCT CCG AGG GGT GCG GAG TCT GTA CAC AGC TTC TAC CAT GCT GAC
--- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- ---
 S   G   G   P   P   R   G   A   E   S   V   H   S   F   Y   H   A   D

         1846        1855        1864        1873        1882        1891
TGC CAC TTG GAG CCA GTC CGT TGC CAG GCA CCC CCT CCC AGA TGC CCA TCG GAG
--- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- ---
 C   H   L   E   P   V   R   C   Q   A   P   P   P   R   C   P   S   E

         1900        1909        1918        1927        1936        1945
GCA TCT GGT AGG ACT GTG GGT AGT GGG AAG GTG TAC CCC ACT GTG CAT ACC AGC
--- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- ---
 A   S   G   R   T   V   G   S   G   K   V   Y   P   T   V   H   T   S

         1954        1963        1972        1981        1990        1999
CCT CCA CCA GAG ATA CTG AAG GAT AAA GCA CTA GTG GAG GTG GCC CCC AGC CCT
--- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- ---
 P   P   P   E   I   L   K   D   K   A   L   V   E   V   A   P   S   P

         2008        2017        2026        2035        2044        2053
GGG CCC CCC ACC CTC ACC AGC TTC AAC ATC CCA CCT GGG CCC TTC AGC TCC ATG
--- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- ---
 G   P   P   T   L   T   S   F   N   I   P   P   G   P   F   S   S   M
```

H:\004\00210\1

DNASIS   DNA Translation [T-INS]

```
         2062        2071        2080        2089        2098        2107
CAC AAG CTC CTG GAG ACA CAG AGT ACG GGA GCC TGC CAT AGC TCC TGC AAA ATC
--- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- ---
 H   K   L   L   E   T   Q   S   T   G   A   C   H   S   S   C   K   I

         2116        2125        2134        2143        2152        2161
TCC AGC CCT TGC TCC AAG GCA GAC AGT GGA GCC TGC GGG CCG GAC AGT TGT CCC
--- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- ---
 S   S   P   C   S   K   A   D   S   G   A   C   G   P   D   S   C   P

         2170        2179        2188        2197        2206        2215
TAC TGT GCC CGG ACA GGA GCA GGA GAG CCA GAG TCC GCT GAC CAT GTC ATG CCT
--- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- ---
 Y   C   A   R   T   G   A   G   E   P   E   S   A   D   H   V   M   P

         2224        2233        2242        2251        2260        2269
GAC TCA GAC AGC GAG GCT GTG TAT GAG TTC ACA CAG GAC GCT CAG CAC AGT GAC
--- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- ---
 D   S   D   S   E   A   V   Y   E   F   T   Q   D   A   Q   H   S   D

         2278        2287        2296        2305        2314        2323
CTC CGG GAT CCC CAC AGC CGG CGG CGA CAG CGG AGC CTG GGC CCA GAT GCA GAG
--- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- ---
 L   R   D   P   H   S   R   R   R   Q   R   S   L   G   P   D   A   E

         2332        2341        2350        2359        2368        2377
CCT AGT TCT GTG CTG GCT TTC TGG AGG CTG ATC TGT GAC ACA TTC CGG AAG ATC
--- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- ---
 P   S   S   V   L   A   F   W   R   L   I · C   D   T   F   R   K   I

         2386        2395        2404        2413        2422        2431
GTA GAT AGC AAA TAC TTT GGC CGG GGA ATC ATG ATC GCC ATC CTG GTC AAT ACA
--- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- ---
 V   D   S   K   Y   F   G   R   G   I   M   I   A   I   L   V   N   T

         2440        2449        2458        2467        2476        2485
CTC AGC ATG GGC ATC GAG TAC CAC GAG CAG CCC GAG GAG CTC ACC AAC GCC CTG
--- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- ---
 L   S   M   G   I   E   Y   H   E   Q   P   E   E   L   T   N   A   L

         2494        2503        2512        2521        2530        2539
GAA ATC AGC AAC ATC GTC TTC ACC AGC CTC TTC GCC TTG GAG ATG CTG CTG AAA
--- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- ---
 E   I   S   N   I   V   F   T   S   L   F   A   L   E   M   L   L   K

         2548        2557        2566        2575        2584        2593
CTG CTT GTC TAC GGT CCC TTT GGC TAC ATT AAG AAT CCC TAC AAC ATC TTT GAT
--- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- ---
 L   L   V   Y   G   P   F   G   Y   I   K   N   P   Y   N   I   F   D

         2602        2611        2620        2629        2638        2647
GGT GTC ATT GTG GTC ATC AGT GTG TGG GAG ATT GTG GGC CAG CAG GGA GGT GGC
--- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- ---
 G   V   I   V   V   I   S   V   W   E   I   V   G   Q   Q   G   G   G

         2656        2665        2674        2683        2692        2701
CTG TCG GTG CTG CGG ACC TTC CGC CTG ATG CGG GTG CTG AAG CTG GTG CGC TTC
--- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- ---
 L   S   V   L   R   T   F   R   L   M   R   V   L   K   L   V   R   F

         2710        2719        2728        2737        2746        2755
CTG CCG GCC CTG CAG CGC CAG CTC GTG GTG CTC ATG AAG ACC ATG GAC AAC GTG
--- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- ---
 L   P   A   L   Q   R   Q   L   V   V   L   M   K   T   M   D   N   V
```

DNASIS   DNA Translation [T-INS]

```
          2764             2773             2782             2791             2800             2809
GCC ACC TTC TGC ATG CTC CTC ATG CTG TTC ATC TTC ATC TTC AGC ATC CTG GGC
--- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- ---
 A   T   F   C   M   L   L   M   L   F   I   F   I   F   S   I   L   G

          2818             2827             2836             2845             2854             2863
ATG CAT CTC TTT GGT TGC AAG TTC GCA TCT GAA CGG GAT GGG GAC ACG TTG CCA
--- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- ---
 M   H   L   F   G   C   K   F   A   S   E   R   D   G   D   T   L   P

          2872             2881             2890             2899             2908             2917
GAC CGG AAG AAT TTC GAC TCC CTG CTC TGG GCC ATC GTC ACT GTC TTT CAG ATT
--- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- ---
 D   R   K   N   F   D   S   L   L   W   A   I   V   T   V   F   Q   I

          2926             2935             2944             2953             2962             2971
CTG ACT CAG GAA GAC TGG AAT AAA GTC CTC TAC AAC GGC ATG GCC TCC ACA TCG
--- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- ---
 L   T   Q   E   D   W   N   K   V   L   Y   N   G   M   A   S   T   S

          2980             2989             2998             3007             3016             3025
TCT TGG GCT GCT CTT TAC TTC ATC GCC CTC ATG ACT TTT GGC AAC TAT GTG CTC
--- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- ---
 S   W   A   A   L   Y   F   I   A   L   M   T   F   G   N   Y   V   L

          3034             3043             3052             3061             3070             3079
TTT AAC CTG CTG GTG GCC ATT CTT GTG GAA GGA TTC CAG GCA GAG GAA ATC GGC
--- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- ---
 F   N   L   L   V   A   I   L   V   E   G   F   Q   A   E   E   I   G

          3088             3097             3106             3115             3124             3133
AAA CGG GAA GAT GCG AGT GGA CAG TTA AGC TGT ATT CAG CTG CCT GTC AAC TCT
--- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- ---
 K   R   E   D   A   S   G   Q   L   S   C   I   Q   L   P   V   N   S

          3142             3151             3160             3169             3178             3187
CAG GGG GGA GAT GCC ACC AAG TCT GAG TCA GAG CCT GAT TTC TTT TCG CCC AGT
--- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- ---
 Q   G   G   D   A   T   K   S   E   S   E   P   D   F   F   S   P   S

          3196             3205             3214             3223             3232             3241
GTG GAT GGT GAT GGG GAC AGA AAG AAG CGC TTG GCC CTG GTG GCT TTG GGA GAA
--- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- ---
 V   D   G   D   G   D   R   K   K   R   L   A   L   V   A   L   G   E

          3250             3259             3268             3277             3286             3295
CAC GCG GAA CTA CGA AAG AGC CTT TTG CCA CCC CTC ATC ATC CAT ACG GCT GCG
--- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- ---
 H   A   E   L   R   K   S   L   L   P   P   L   I   I   H   T   A   A

          3304             3313             3322             3331             3340             3349
ACA CCA ATG TCA CTA CCC AAG AGC TCC AGC ACA GGT GTG GGG GAA GCA CTG GGC
--- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- ---
 T   P   M   S   L   P   K   S   S   S   T   G   V   G   E   A   L   G

          3358             3367             3376             3385             3394             3403
TCT GGC TCT CGA CGT ACC AGT AGC AGT GGG TCC GCT GAG CCT GGA GCT GCC CAC
--- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- ---
 S   G   S   R   R   T   S   S   S   G   S   A   E   P   G   A   A   H

          3412             3421             3430             3439             3448             3457
CAT GAG ATG AAA TCT CCG CCA AGT GCC CGC AGC TCC CCG CAC AGT CCC TGG AGT
--- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- ---
 H   E   M   K   S   P   P   S   A   R   S   S   P   H   S   P   W   S
```

DNASIS   DNA Translation [T-INS]

```
         3466          3475          3484          3493          3502          3511
GCG GCA AGC AGC TGG ACC AGC AGG CGC TCC AGC AGG AAC AGC CTG GGC CGG GCC
--- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- ---
 A   A   S   S   W   T   S   R   R   S   S   R   N   S   L   G   R   A

         3520          3529          3538          3547          3556          3565
CCC AGC CTA AAG CGG AGG AGC CCG AGC GGG GAG CGG AGG TCC CTG CTG TCT GGA
--- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- ---
 P   S   L   K   R   R   S   P   S   G   E   R   R   S   L   L   S   G

         3574          3583          3592          3601          3610          3619
GAG GGC CAG GAG AGT CAG GAT GAG GAG GAA AGT TCA GAA GAG GAC CGG GCC AGC
--- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- ---
 E   G   Q   E   S   Q   D   E   E   S   S   E   D   R   A   S

         3628          3637          3646          3655          3664          3673
CCA GCA GGC AGT GAC CAT CGC CAC AGG GGT TCC TTG GAA CGT GAG GCC AAG AGT
--- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- ---
 P   A   G   S   D   H   R   H   R   G   S   L   E   R   E   A   K   S

         3682          3691          3700          3709          3718          3727
TCC TTT GAC CTG CCT GAC ACT CTG CAG GTG CCG GGG CTG CAC CGC ACA GCC AGC
--- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- ---
 S   F   D   L   P   D   T   L   Q   V   P   G   L   H   R   T   A   S

         3736          3745          3754          3763          3772          3781
GGC CGG AGC TCT GCC TCT GAG CAC CAA GAC TGT AAT GGC AAG TCG GCT TCA GGG
--- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- ---
 G   R   S   S   A   S   E   H   Q   D   C   N   G   K   S   A   S   G

         3790          3799          3808          3817          3826          3835
CGT TTG GCC CGC ACC CTG AGG ACT GAT GAC CCC CAA CTG GAT GGG GAT GAT GAC
--- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- ---
 R   L   A   R   T   L   R   T   D   D   P   Q   L   D   G   D   D   D

         3844          3853          3862          3871          3880          3889
AAT GAT GAG GGA AAT CTG AGC AAA GGG GAA CGC ATA CAA GCC TGG GTC AGA TCC
--- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- ---
 N   D   E   G   N   L   S   K   G   E   R   I   Q   A   W   V   R   S

         3898          3907          3916          3925          3934          3943
CGG CTT CCT GCC TGT TGC CGA GAG CGA GAT TCC TGG TCG GCC TAT ATC TTT CCT
--- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- ---
 R   L   P   A   C   C   R   E   R   D   S   W   S   A   Y   I   F   P

         3952          3961          3970          3979          3988          3997
CCT CAG TCA AGG TTT CGT CTC CTG TGT CAC CGG ATC ATC ACC CAC AAG ATG TTT
--- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- ---
 P   Q   S   R   F   R   L   L   C   H   R   I   I   T   H   K   M   F

         4006          4015          4024          4033          4042          4051
GAC CAT GTG GTC CTC GTC ATC ATC TTC CTC AAC TGT ATC ACC ATC GCT ATG GAG
--- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- ---
 D   H   V   V   L   V   I   I   F   L   N   C   I   T   I   A   M   E

         4060          4069          4078          4087          4096          4105
CGC CCC AAA ATT GAC CCC CAC AGC GCT GAG CGC ATC TTC CTG ACC CTC TCC AAC
--- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- ---
 R   P   K   I   D   P   H   S   A   E   R   I   F   L   T   L   S   N

         4114          4123          4132          4141          4150          4159
TAC ATC TTC ACG GCA GTC TTT CTA GCT GAA ATG ACA GTG AAG GTG GTG GCA CTG
--- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- ---
 Y   I   F   T   A   V   F   L   A   E   M   T   V   K   V   V   A   L
```

DNASIS   DNA Translation [T-INS]

```
        4168            4177            4186            4195            4204            4213
GGC TGG TGC TTT GGG GAG CAG GCC TAC CTG CGC AGC AGC TGG AAT GTG CTG GAC
--- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- ---
 G   W   C   F   G   E   Q   A   Y   L   R   S   S   W   N   V   L   D

        4222            4231            4240            4249            4258            4267
GGC TTG CTG GTG CTC ATC TCC GTC ATC GAC ATC CTG GTC TCC ATG GTC TCC GAC
--- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- ---
 G   L   L   V   L   I   S   V   I   D   I   L   V   S   M   V   S   D

        4276            4285            4294            4303            4312            4321
AGC GGC ACC AAG ATC CTT GGC ATG CTG AGG GTG CTG CGG CTG CTG CGG ACC CTG
--- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- ---
 S   G   T   K   I   L   G   M   L   R   V   L   R   L   L   R   T   L

        4330            4339            4348            4357            4366            4375
CGT CCA CTC AGG GTC ATC AGC CGG GCC CAG GGA CTG AAG CTG GTG GTA GAG ACT
--- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- ---
 R   P   L   R   V   I   S   R   A   Q   G   L   K   L   V   V   E   T

        4384            4393            4402            4411            4420            4429
CTG ATG TCA TCC CTC AAA CCC ATT GGC AAC ATT GTG GTC ATT TGC TGT GCC TTC
--- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- ---
 L   M   S   S   L   K   P   I   G   N   I   V   V   I   C   C   A   F

        4438            4447            4456            4465            4474            4483
TTC ATC ATT TTT GGA ATT CTC GGG GTG CAG CTC TTC AAA GGG AAG TTC TTC GTG
--- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- ---
 F   I   I   F   G   I   L   G   V   Q   L   F   K   G   K   F   F   V

        4492            4501            4510            4519            4528            4537
TGT CAG GGT GAG GAC ACC AGG AAC ATC ACT AAC AAA TCC GAC TGC GCT GAG GCC
--- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- ---
 C   Q   G   E   D   T   R   N   I   T   N   K   S   D   C   A   E   A

        4546            4555            4564            4573            4582            4591
AGC TAC CGA TGG GTC CGG CAC AAG TAC AAC TTT GAC AAC CTG GGC CAG GCT CTG
--- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- ---
 S   Y   R   W   V   R   H   K   Y   N   F   D   N   L   G   Q   A   L

        4600            4609            4618            4627            4636            4645
ATG TCC CTG TTT GTG CTG GCC TCC AAG GAT GGT TGG GTT GAC ATC ATG TAT GAT
--- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- ---
 M   S   L   F   V   L   A   S   K   D   G   W   V   D   I   M   Y   D

        4654            4663            4672            4681            4690            4699
GGG CTG GAT GCT GTG GGT GTG GAT CAG CAG CCC ATC ATG AAC CAC AAC CCC TGG
--- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- ---
 G   L   D   A   V   G   V   D   Q   Q   P   I   M   N   H   N   P   W

        4708            4717            4726            4735            4744            4753
ATG CTG CTA TAC TTC ATC TCC TTC CTC CTC ATC GTG GCC TTC TTT GTC CTG AAC
--- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- ---
 M   L   L   Y   F   I   S   F   L   L   I   V   A   F   F   V   L   N

        4762            4771            4780            4789            4798            4807
ATG TTT GTG GGC GTG GTG GTG GAG AAC TTC CAT AAG TGC AGA CAG CAC CAG GAG
--- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- ---
 M   F   V   G   V   V   V   E   N   F   H   K   C   R   Q   H   Q   E

        4816            4825            4834            4843            4852            4861
GAG GAG GAG GCG AGG CGG CGT GAG GAG AAG CGA CTA CGG AGG CTG GAG AAA AAG
--- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- ---
 E   E   E   A   R   R   R   E   E   K   R   L   R   R   L   E   K   K
```

DNASIS DNA Translation [T-INS]

```
     4870          4879          4888          4897          4906          4915
AGA AGG AAT CTA ATG TTG GAC GAT GTA ATT GCT TCC GGC AGC TCA GCC AGC GCT
--- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- ---
 R   R   N   L   M   L   D   D   V   I   A   S   G   S   S   A   S   A

     4924          4933          4942          4951          4960          4969
GCG TCA GAA GCC CAG TGC AAG CCC TAC TAC TCT GAC TAC TCG AGA TTC CGG CTC
--- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- ---
 A   S   E   A   Q   C   K   P   Y   Y   S   D   Y   S   R   F   R   L

     4978          4987          4996          5005          5014          5023
CTT GTC CAC CAC CTG TGT ACC AGC CAC TAC CTG GAC CTC TTC ATC ACT GGT GTC
--- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- ---
 L   V   H   H   L   C   T   S   H   Y   L   D   L   F   I   T   G   V

     5032          5041          5050          5059          5068          5077
ATC GGG CTG AAC GTG GTC ACT ATG GCC ATG GAA CAT TAC CAG CAG CCC CAG ATC
--- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- ---
 I   G   L   N   V   V   T   M   A   M   E   H   Y   Q   Q   P   Q   I

     5086          5095          5104          5113          5122          5131
CTG GAC GAG GCT CTG AAG ATC TGC AAT TAC ATC TTT ACC GTC ATC TTT GTC TTT
--- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- ---
 L   D   E   A   L   K   I   C   N   Y   I   F   T   V   I   F   V   F

     5140          5149          5158          5167          5176          5185
GAG TCA GTT TTC AAA CTT GTG GCC TTT GGC TTC CGC CGT TTC TTC CAG GAC AGG
--- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- ---
 E   S   V   F   K   L   V   A   F   G   F   R   R   F   F   Q   D   R

     5194          5203          5212          .5221         5230          5239
TGG AAC CAG CTG GAC CTG GCT ATT GTG CTT CTG TCC ATC ATG GGC ATC ACA CTG
--- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- ---
 W   N   Q   L   D   L   A   I   V   L   L   S   I   M   G   I   T   L

     5248          5257          5266          5275          5284          5293
GAG GAG ATT GAG GTC AAT GCT TCG CTG CCC ATC AAC CCC ACC ATC ATC CGT ATC
--- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- ---
 E   E   I   E   V   N   A   S   L   P   I   N   P   T   I   I   R   I

     5302          5311          5320          .5329         5338          5347
ATG AGG GTG CTC CGC ATT GCT CGA GTT CTG AAG CTG TTG AAG ATG GCT GTG GGC
--- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- ---
 M   R   V   L   R   I   A   R   V   L   K   L   L   K   M   A   V   G

     5356          5365          5374          5383          5392          5401
ATG CGG GCA CTG CTG GAC ACG GTG ATG CAG GCC CTG CCC CAG GTG GGG AAC CTG
--- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- ---
 M   R   A   L   L   D   T   V   M   Q   A   L   P   Q   V   G   N   L

     5410          5419          5428          5437          5446          5455
GGA CTT CTC TTC ATG TTA TTG TTT TTC ATC TTT GCA GCT CTG GGC GTG GAG CTC
--- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- ---
 G   L   L   F   M   L   L   F   F   I   F   A   A   L   G   V   E   L

     5464          5473          5482          5491          5500          5509
TTT GGA GAC CTG GAG TGT GAT GAG ACA CAC CCT TGT GAG GGC TTG GGT CGG CAT
--- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- ---
 F   G   D   L   E   C   D   E   T   H   P   C   E   G   L   G   R   H

     5518          5527          5536          5545          5554          5563
GCC ACC TTT AGG AAC TTT GGT ATG GCC TTT CTG ACC CTC TTC CGA GTC TCC ACT
--- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- ---
 A   T   F   R   N   F   G   M   A   F   L   T   L   F   R   V   S   T
```

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DNASIS   DNA Translation [T-INS]

```
        5572          5581          5590          5599          5608          5617
GGT GAC AAC TGG AAT GGT ATT ATG AAG GAC ACC CTC CGG GAC TGT GAC CAG GAG
--- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- ---
 G   D   N   W   N   G   I   M   K   D   T   L   R   D   C   D   Q   E

        5626          5635          5644          5653          5662          5671
TCC ACC TGC TAC AAC ACT GTC ATC TCC CCT ATC TAC TTT GTG TCC TTC GTG CTG
--- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- ---
 S   T   C   Y   N   T   V   I   S   P   I   Y   F   V   S   F   V   L

        5680          5689          5698          5707          5716          5725
ACG GCC CAG TTT GTG CTG GTC AAC GTG GTC ATA GCT GTG CTG ATG AAG CAC CTG
--- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- ---
 T   A   Q   F   V   L   V   N   V   V   I   A   V   L   M   K   H   L

        5734          5743          5752          5761          5770          5779
GAA GAA AGC AAC AAA GAG GCC AAG GAG GAG GCC GAG CTC GAG GCC GAG CTG GAG
--- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- ---
 E   E   S   N   K   E   A   K   E   E   A   E   L   E   A   E   L   E

        5788          5797          5806          5815          5824          5833
CTG GAG ATG AAG ACG CTC AGC CCG CAG CCC CAC TCC CCG CTG GGC AGC CCC TTC
--- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- ---
 L   E   M   K   T   L   S   P   Q   P   H   S   P   L   G   S   P   F

        5842          5851          5860          5869          5878          5887
CTC TGG CCC GGG GTG GAG GGT GTC AAC AGT CCT GAC AGC CCT AAG CCT GGG GCT
--- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- ---
 L   W   P   G   V   E   G   V   N   S   P   D   S   P   K   P   G   A

        5896          5905          5914          5923          5932          5941
CCA CAC ACC ACT GCC CAC ATT GGA GCA GCC TCG GGC TTC TCC CTT GAG CAC CCC
--- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- ---
 P   H   T   T   A   H   I   G   A   A   S   G   F   S   L   E   H   P

        5950          5959          5968          5977          5986          5995
ACG ATG GTA CCC CAC CCC GAG GAG GTG CCA GTC CCC CTA GGA CCA GAC CTG CTG
--- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- ---
 T   M   V   P   H   P   E   E   V   P   V   P   L   G   P   D   L   L

        6004          6013          6022          6031          6040          6049
ACT GTG AGG AAG TCT GGT GTC AGC CGG ACG CAC TCT CTG CCC AAT GAC AGC TAC
--- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- ---
 T   V   R   K   S   G   V   S   R   T   H   S   L   P   N   D   S   Y

        6058          6067          6076          6085          6094          6103
ATG TGC CGC AAT GGG AGC ACT GCT GAG AGA TCC CTA GGA CAC AGG GGC TGG GGG
--- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- ---
 M   C   R   N   G   S   T   A   E   R   S   L   G   H   R   G   W   G

        6112          6121          6130          6139          6148          6157
CTC CCC AAA GCC CAG TCA GGC TCC ATC TTG TCC GTT CAC TCC CAA CCA GCA GAC
--- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- ---
 L   P   K   A   Q   S   G   S   I   L   S   V   H   S   Q   P   A   D

        6166          6175          6184          6193          6202          6211
ACC AGC TGC ATC CTA CAG CTT CCC AAA GAT GTG CAC TAT CTG CTC CAG CCT CAT
--- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- ---
 T   S   C   I   L   Q   L   P   K   D   V   H   Y   L   L   Q   P   H

        6220          6229          6238          6247          6256          6265
GGG GCC CCC ACC TGG GGC GCC ATC CCT AAA CTA CCC CCA CCT GGC CGC TCC CCT
--- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- ---
 G   A   P   T   W   G   A   I   P   K   L   P   P   P   G   R   S   P
```

DNASIS   DNA Translation [T-INS]

```
         6274           6283           6292           6301           6310           6319
CTG GCT CAG AGG CCT CTC AGG CGC CAG GCA GCA ATA AGG ACT GAC TCC CTG GAT
--- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- ---
 L   A   Q   R   P   L   R   R   Q   A   A   I   R   T   D   S   L   D

         6328           6337           6346           6355           6364           6373
GTG CAG GGC CTG GGT AGC CGG GAA GAC CTG TTG TCA GAG GTG AGT GGG CCC TCC
--- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- ---
 V   Q   G   L   G   S   R   E   D   L   L   S   E   V   S   G   P   S

         6382           6391           6400           6409           6418           6427
TGC CCT CTG ACC CGG TCC TCA TCC TTC TGG GGC GGG TCG AGC ATC CAG GTG CAG
--- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- ---
 C   P   L   T   R   S   S   S   F   W   G   G   S   S   I   Q   V   Q

         6436           6445           6454           6463           6472           6481
CAG CGT TCC GGC ATC CAG AGC AAA GTC TCC AAG CAC ATC CGC CTG CCA GCC CCT
--- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- ---
 Q   R   S   G   I   Q   S   K   V   S   K   H   I   R   L   P   A   P

         6490           6499           6508           6517           6526           6535
TGC CCA GGC CTG GAA CCC AGC TGG GCC AAG GAC CCT CCA GAG ACC AGA AGC AGC
--- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- ---
 C   P   G   L   E   P   S   W   A   K   D   P   P   E   T   R   S   S

         6544           6553           6562           6571           6580           6589
TTA GAG CTG GAC ACG GAG CTG AGC TGG ATT TCA GGA GAC CTC CTT CCC AGC AGC
--- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- ---
 L   E   L   D   T   E   L   S   W   I   S   G   D   L   L   P   S   S

         6598           6607           6616           6625           6634           6643
CAG GAA GAA CCC CTG TCC CCA CGG GAC CTG AAG AAG TGC TAC AGT GTA GAG ACC
--- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- ---
 Q   E   E   P   L   S   P   R   D   L   K   K   C   Y   S   V   E   T

         6652           6661           6670           6679           6688           6697
CAG AGC TGC AGG CGC AGG CCT GGG TCC TGG CTA GAT GAA CAG CGG AGA CAC TCC
--- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- ---
 Q   S   C   R   R   R   P   G   S   W   L   D   E   Q   R   R   H   S

         6706           6715           6724           6733           6742           6751
ATT GCT GTC AGC TGT CTG GAC AGC GGC TCC CAA CCC CGC CTA TGT CCA AGC CCC
--- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- ---
 I   A   V   S   C   L   D   S   G   S   Q   P   R   L   C   P   S   P

         6760           6769           6778           6787           6796           6805
TCA AGC CTC GGG GGC CAA CCT CTT GGG GGT CCT GGG AGC CGG CCT AAG AAA AAA
--- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- ---
 S   S   L   G   G   Q   P   L   G   G   P   G   S   R   P   K   K   K

         6814           6823           6832           6841           6850           6859
CTC AGC CCA CCC AGT ATC TCT ATA GAC CCC CCG GAG AGC CAG GGC TCT CGG CCC
--- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- ---
 L   S   P   P   S   I   S   I   D   P   P   E   S   Q   G   S   R   P

         6868           6877           6886           6895           6904           6913
CCA TGC AGT CCT GGT GTC TGC CTC AGG AGG AGG GCG CCG GCC AGT GAC TCT AAG
--- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- ---
 P   C   S   P   G   V   C   L   R   R   R   A   P   A   S   D   S   K

         6922           6931           6940           6949           6958           6967
GAT CCC TCG GTC TCC AGC CCC CTT GAC AGC ACG GCT GCC TCA CCC TCC CCA AAG
--- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- ---
 D   P   S   V   S   S   P   L   D   S   T   A   A   S   P   S   P   K
```

DNASIS   DNA Translation [T-INS]

```
        6976        6985        6994        7003        7012        7021
AAA GAC ACG CTG AGT CTC TCT GGT TTG TCT TCT GAC CCA ACA GAC ATG GAC CCC
--- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- ---
 K   D   T   L   S   L   S   G   L   S   S   D   P   T   D   M   D   P

        7030        7039        7048        7057        7066        7075
TGA GTC CTA CCC ACT CTC CCC CAT CAC CTT TCT CCA CCG GGT GCA GAT CCT AGC
--- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- ---
 *   V   L   P   T   L   P   H   H   L   S   P   P   G   A   D   P   S

        7084        7093        7102        7111        7120        7129
TCC GCC TCC TGG GCA GCG TTT CTG AAA AGT CCC ACG TAA GCA GCA AGC AGC CAC
--- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- ---
 S   A   S   W   A   A   F   L   K   S   P   T   *   A   A   S   S   H

        7138        7147        7156        7165        7174        7183
GAG GCA CCT CAC CTG CCT TCT TCA GTG GCT GGT GGG GAT GAC GAG CAG AAC TTC
--- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- ---
 E   A   P   H   L   P   S   S   V   A   G   G   D   D   E   Q   N   F

        7192        7201        7210        7219        7228        7237
CGG AGA GTC GAT CTG AAG AGA ACA CAG CCC TGG AGC CCC TGC CTC CGG GAA GAA
--- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- ---
 R   R   V   D   L   K   R   T   Q   P   W   S   P   C   L   R   E   E

        7246        7255        7264        7273        7282
GGA AAA GGA GAA AGC CCA GTG TGG CCA AGG CTC CCG ACA CCA GGA GCT G/3'
--- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- ---
 G   K   G   E   S   P   V   W   P   R   L   P   T   P   G   A/
```

↙ SEQ 1 + 3

↙ SEQ 2 + 4

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DNASIS
T-INS
      SEQ ID NO: 3
       10          20          30          40          50          60
GGAGCTGAGC  TGAACTGGCC  CTCCTGGGGA  CTCAGCAAGC  TCTCTAGAGC  CCCCCACATG

       70          80          90         100         110         120
CTCCCCCACC  GGGGTCCCCC  GGTTGCGTGA  GGACACCTCC  TCTGAGGGGC  TCCGCTCGCC

                                                                         Start of
      130         140         150         160         170         180    SEQ ID NO: 1
CCTCTTCGGA  CCCCCCGGGG  CCCCGGCTGG  CCAGAGGATG  GACGAGGAGG  AGGATGGAGC   coding region

      190         200         210         220         230         240
GGGCGCCGAG  GAGTCGGGAC  AGCCCCGTAG  CTTCACGCAG  CTCAACGACC  TGTCCGGGGC

      250         260         270         280         290         300
CGGGGGCCGG  CAGGGGCCGG  GGTCGACGGA  AAAGGACCCG  GGCAGCGCGG  ACTCCGAGGC

      310         320         330         340         350         360
GGAGGGGCTG  CCGTACCCGG  CGCTAGCCCC  GGTGGTTTTC  TTCTACTTGA  GCCAGGACAG

      370         380         390         400         410         420
CCGCCCGCGG  AGCTGGTGTC  TCCGCACGGT  CTGTAACCCG  TGGTTCGAGC  GAGTCAGTAT

      430         440         450         460         470         480
GCTGGTCATT  CTTCTCAACT  GTGTGACTCT  GGGTATGTTC  AGGCCGTGTG  AGGACATTGC

      490         500         510         520         530         540
CTGTGACTCC  CAGCGCTGCC  GGATCCTGCA  GGCCTTCGAT  GACTTCATCT  TTGCCTTCTT

      550         560         570         580         590         600
TGCTGTGGAA  ATGGTGGTGA  AGATGGTGGC  CTTGGGCATC  TTTGGGAAGA  AATGTTACCT

      610         620         630         640         650         660
GGGAGACACT  TGGAACCGGC  TTGACTTTTT  CATTGTCATT  GCAGGGATGC  TGGAGTATTC

      670         680         690         700         710         720
GCTGGACCTG  CAGAACGTCA  GCTTCTCCGC  AGTCAGGACA  GTCCGTGTGC  TGCGACCGCT

      730         740         750         760         770         780
CAGGGCCATT  AACCGGGTGC  CCAGCATGCG  CATTCTCGTC  ACATTACTGC  TGGACACCTT

      790         800         810         820         830         840
GCCTATGCTG  GGCAACGTCC  TGCTGCTCTG  TTTCTTCGTC  TTTTTCATCT  TTGGCATCGT

      850         860         870         880         890         900
GGGCGTCCAG  CTGTGGGCAG  GACTGCTTCG  CAACCGATGC  TTCCTCCCCG  AGAACTTCAG

      910         920         930         940         950         960
CCTCCCCCTG  AGCGTGGACC  TGGAGCCTTA  TTACCAGACA  GAGAATGAGG  ACGAGAGCCC

      970         980         990        1000        1010        1020
CTTCATCTGC  TCTCAGCCTC  GGGAGAATGG  CATGAGATCC  TGCAGGAGTG  TGCCCACACT

     1030        1040        1050        1060        1070        1080
GCGTGGGGAA  GGCGGTGGTG  GCCCACCCTG  CAGTCTGGAC  TATGAGACCT  ATAACAGTTC

     1090        1100        1110        1120        1130        1140
CAGCAACACC  ACCTGTGTCA  ACTGGAACCA  GTACTATACC  AACTGCTCTG  CGGGCGAGCA

     1150        1160        1170        1180        1190        1200
CAACCCCTTC  AAAGGCGCCA  TCAACTTTGA  CAACATTGGC  TATGCCTGGA  TCGCCATCTT

     1210        1220        1230        1240        1250        1260
CCAGGTCATC  ACACTGGAGG  GCTGGGTCGA  CATCATGTAC  TTCGTAATGG  ACGCTCACTC
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DNASIS
T-INS

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           1270       1280       1290       1300       1310       1320
     CTTCTACAAC TTCATCTACT TCATTCTTCT CATCATCGTG GGCTCCTTCT TCATGATCAA

           1330       1340       1350       1360       1370       1380
     CCTGTGCCTG GTGGTGATTG CCACGCAGTT CTCCGAGACC AAACAGCGGG AGAGTCAGCT

           1390       1400       1410       1420       1430       1440
     GATGCGGGAG CAGCGTGTAC GATTCCTGTC CAATGCTAGC ACCCTGGCAA GCTTCTCTGA

           1450       1460       1470       1480       1490       1500
     GCCAGGCAGC TGCTATGAGG AGCTACTCAA GTACCTGGTG TACATCCTCC GAAAAGCAGC

           1510       1520       1530       1540       1550       1560
     CCGAAGGCTG GCCCAGGTCT CTAGGGCTAT AGGCGTGCGG GCTGGGCTGC TCAGCAGCCC

           1570       1580       1590       1600       1610       1620
     AGTGGCCCGT AGTGGGCAGG AGCCCCAGCC CAGTGGCAGC TGCACTCGCT CACACCGTCG

           1630       1640       1650       1660       1670       1680
     TCTGTCTGTC CACCACCTGG TCCACCACCA TCACCACCAC CATCACCACT ACCACCTGGG

           1690       1700       1710       1720       1730       1740
     TAATGGGACG CTCAGAGTTC CCCGGGCCAG CCCAGAGATC CAGGACAGGG ATGCCAATGG

           1750       1760       1770       1780       1790       1800
     GTCTCGCCGG CTCATGCTAC CACCACCCTC TACACCCACT CCCTCTGGGG GCCCTCCGAG

           1810       1820       1830       1840       1850       1860
     GGGTGCGGAG TCTGTACACA GCTTCTACCA TGCTGACTGC CACTTGGAGC CAGTCCGTTG

           1870       1880       1890       1900       1910       1920
     CCAGGCACCC CCTCCCAGAT GCCCATCGGA GGCATCTGGT AGGACTGTGG GTAGTGGGAA

           1930       1940       1950       1960       1970       1980
     GGTGTACCCC ACTGTGCATA CCAGCCCTCC ACCAGAGATA CTGAAGGATA AAGCACTAGT

           1990       2000       2010       2020       2030       2040
     GGAGGTGGCC CCCAGCCCTG GGCCCCCCAC CCTCACCAGC TTCAACATCC CACCTGGGCC

           2050       2060       2070       2080       2090       2100
     CTTCAGCTCC ATGCACAAGC TCCTGGAGAC ACAGAGTACG GGAGCCTGCC ATAGCTCCTG

           2110       2120       2130       2140       2150       2160
     CAAAATCTCC AGCCCTTGCT CCAAGGCAGA CAGTGGAGCC TGCGGGCCGG ACAGTTGTCC

           2170       2180       2190       2200       2210       2220
     CTACTGTGCC CGGACAGGAG CAGGAGAGCC AGAGTCCGCT GACCATGTCA TGCCTGACTC

           2230       2240       2250       2260       2270       2280
     AGACAGCGAG GCTGTGTATG AGTTCACACA GGACGCTCAG CACAGTGACC TCCGGGATCC

           2290       2300       2310       2320       2330       2340
     CCACAGCCGG CGGCGACAGC GGAGCCTGGG CCCAGATGCA GAGCCTAGTT CTGTGCTGGC

           2350       2360       2370       2380       2390       2400
     TTTCTGGAGG CTGATCTGTG ACACATTCCG GAAGATCGTA GATAGCAAAT ACTTTGGCCG

           2410       2420       2430       2440       2450       2460
     GGGAATCATG ATCGCCATCC TGGTCAATAC ACTCAGCATG GGCATCGAGT ACCACGAGCA

           2470       2480       2490       2500       2510       2520
     GCCCGAGGAG CTCACCAACG CCCTGGAAAT CAGCAACATC GTCTTCACCA GCCTCTTCGC
```

DNASIS
T-INS

```
       2530        2540        2550        2560        2570        2580
CTTGGAGATG  CTGCTGAAAC  TGCTTGTCTA  CGGTCCCTTT  GGCTACATTA  AGAATCCCTA

       2590        2600        2610        2620        2630        2640
CAACATCTTT  GATGGTGTCA  TTGTGGTCAT  CAGTGTGTGG  GAGATTGTGG  GCCAGCAGGG

       2650        2660        2670        2680        2690        2700
AGGTGGCCTG  TCGGTGCTGC  GGACCTTCCG  CCTGATGCGG  GTGCTGAAGC  TGGTGCGCTT

       2710        2720        2730        2740        2750        2760
CCTGCCGGCC  CTGCAGCGCC  AGCTCGTGGT  GCTCATGAAG  ACCATGGACA  ACGTGGCCAC

       2770        2780        2790        2800        2810        2820
CTTCTGCATG  CTCCTCATGC  TGTTCATCTT  CATCTTCAGC  ATCCTGGGCA  TGCATCTCTT

       2830        2840        2850        2860        2870        2880
TGGTTGCAAG  TTCGCATCTG  AACGGGATGG  GGACACGTTG  CCAGACCGGA  AGAATTTCGA

       2890        2900        2910        2920        2930        2940
CTCCCTGCTC  TGGGCCATCG  TCACTGTCTT  TCAGATTCTG  ACTCAGGAAG  ACTGGAATAA

       2950        2960        2970        2980        2990        3000
AGTCCTCTAC  AACGGCATGG  CCTCCACATC  GTCTTGGGCT  GCTCTTTACT  TCATCGCCCT

       3010        3020        3030        3040        3050        3060
CATGACTTTT  GGCAACTATG  TGCTCTTTAA  CCTGCTGGTG  GCCATTCTTG  TGGAAGGATT

       3070        3080        3090        3100        3110        3120
CCAGGCAGAG  GAAATCGGCA  AACGGGAAGA  TGCGAGTGGA  CAGTTAAGCT  GTATTCAGCT

       3130        3140        3150        3160        3170        3180
GCCTGTCAAC  TCTCAGGGGG  GAGATGCCAC  CAAGTCTGAG  TCAGAGCCTG  ATTTCTTTTC

       3190        3200        3210        3220        3230        3240
GCCCAGTGTG  GATGGTGATG  GGGACAGAAA  GAAGCGCTTG  GCCCTGGTGG  CTTTGGGAGA

       3250        3260        3270        3280        3290        3300
ACACGCGGAA  CTACGAAAGA  GCCTTTTGCC  ACCCCTCATC  ATCCATACGG  CTGCGACACC

       3310        3320        3330        3340        3350        3360
AATGTCACTA  CCCAAGAGCT  CCAGCACAGG  TGTGGGGGAA  GCACTGGGCT  CTGGCTCTCG

       3370        3380        3390        3400        3410        3420
ACGTACCAGT  AGCAGTGGGT  CCGCTGAGCC  TGGAGCTGCC  CACCATGAGA  TGAAATCTCC

       3430        3440        3450        3460        3470        3480
GCCAAGTGCC  CGCAGCTCCC  CGCACAGTCC  CTGGAGTGCG  GCAAGCAGCT  GGACCAGCAG

       3490        3500        3510        3520        3530        3540
GCGCTCCAGC  AGGAACAGCC  TGGGCCGGGC  CCCCAGCCTA  AAGCGGAGGA  GCCCGAGCGG

       3550        3560        3570        3580        3590        3600
GGAGCGGAGG  TCCCTGCTGT  CTGGAGAGGG  CCAGGAGAGT  CAGGATGAGG  AGGAAAGTTC

       3610        3620        3630        3640        3650        3660
AGAAGAGGAC  CGGGCCAGCC  CAGCAGGCAG  TGACCATCGC  CACAGGGGTT  CCTTGGAACG

       3670        3680        3690        3700        3710        3720
TGAGGCCAAG  AGTTCCTTTG  ACCTGCCTGA  CACTCTGCAG  GTGCCGGGGC  TGCACCGCAC

       3730        3740        3750        3760        3770        3780
AGCCAGCGGC  CGGAGCTCTG  CCTCTGAGCA  CCAAGACTGT  AATGGCAAGT  CGGCTTCAGG
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DNASIS
T-INS

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      3790        3800        3810        3820        3830        3840
GCGTTTGGCC  CGCACCCTGA  GGACTGATGA  CCCCCAACTG  GATGGGGATG  ATGACAATGA

      3850        3860        3870        3880        3890        3900
TGAGGGAAAT  CTGAGCAAAG  GGGAACGCAT  ACAAGCCTGG  GTCAGATCCC  GGCTTCCTGC

      3910        3920        3930        3940        3950        3960
CTGTTGCCGA  GAGCGAGATT  CCTGGTCGGC  CTATATCTTT  CCTCCTCAGT  CAAGGTTTCG

      3970        3980        3990        4000        4010        4020
TCTCCTGTGT  CACCGGATCA  TCACCCACAA  GATGTTTGAC  CATGTGGTCC  TCGTCATCAT

      4030        4040        4050        4060        4070        4080
CTTCCTCAAC  TGTATCACCA  TCGCTATGGA  GCGCCCCAAA  ATTGACCCCC  ACAGCGCTGA

      4090        4100        4110        4120        4130        4140
GCGCATCTTC  CTGACCCTCT  CCAACTACAT  CTTCACGGCA  GTCTTTCTAG  CTGAAATGAC

      4150        4160        4170        4180        4190        4200
AGTGAAGGTG  GTGGCACTGG  GCTGGTGCTT  TGGGGAGCAG  GCCTACCTGC  GCAGCAGCTG

      4210        4220        4230        4240        4250        4260
GAATGTGCTG  GACGGCTTGC  TGGTGCTCAT  CTCCGTCATC  GACATCCTGG  TCTCCATGGT

      4270        4280        4290        4300        4310        4320
CTCCGACAGC  GGCACCAAGA  TCCTTGGCAT  GCTGAGGGTG  CTGCGGCTGC  TGCGGACCCT

      4330        4340        4350        4360        4370        4380
GCGTCCACTC  AGGGTCATCA  GCCGGGCCCA  GGGACTGAAG  CTGGTGGTAG  AGACTCTGAT

      4390        4400        4410        4420        4430        4440
GTCATCCCTC  AAACCCATTG  GCAACATTGT  GGTCATTTGC  TGTGCCTTCT  TCATCATTTT

      4450        4460        4470        4480        4490        4500
TGGAATTCTC  GGGGTGCAGC  TCTTCAAAGG  GAAGTTCTTC  GTGTGTCAGG  GTGAGGACAC

      4510        4520        4530        4540        4550        4560
CAGGAACATC  ACTAACAAAT  CCGACTGCGC  TGAGGCCAGC  TACCGATGGG  TCCGGCACAA

      4570        4580        4590        4600        4610        4620
GTACAACTTT  GACAACCTGG  GCCAGGCTCT  GATGTCCCTG  TTTGTGCTGG  CCTCCAAGGA

      4630        4640        4650        4660        4670        4680
TGGTTGGGTT  GACATCATGT  ATGATGGGCT  GGATGCTGTG  GGTGTGGATC  AGCAGCCCAT

      4690        4700        4710        4720        4730        4740
CATGAACCAC  AACCCCTGGA  TGCTGCTATA  CTTCATCTCC  TTCCTCCTCA  TCGTGGCCTT

      4750        4760        4770        4780        4790        4800
CTTTGTCCTG  AACATGTTTG  TGGGCGTGGT  GGTGGAGAAC  TTCCATAAGT  GCAGACAGCA

      4810        4820        4830        4840        4850        4860
CCAGGAGGAG  GAGGAGGCGA  GGCGGCGTGA  GGAGAAGCGA  CTACGGAGGC  TGGAGAAAAA

      4870        4880        4890        4900        4910        4920
GAGAAGGAAT  CTAATGTTGG  ACGATGTAAT  TGCTTCCGGC  AGCTCAGCCA  GCGCTGCGTC

      4930        4940        4950        4960        4970        4980
AGAAGCCCAG  TGCAAGCCCT  ACTACTCTGA  CTACTCGAGA  TTCCGGCTCC  TTGTCCACCA

      4990        5000        5010        5020        5030        5040
CCTGTGTACC  AGCCACTACC  TGGACCTCTT  CATCACTGGT  GTCATCGGGC  TGAACGTGGT
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DNASIS
T-INS

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      5050        5060        5070        5080        5090        5100
CACTATGGCC ATGGAACATT ACCAGCAGCC CCAGATCCTG GACGAGGCTC TGAAGATCTG

      5110        5120        5130        5140        5150        5160
CAATTACATC TTTACCGTCA TCTTTGTCTT TGAGTCAGTT TTCAAACTTG TGGCCTTTGG

      5170        5180        5190        5200        5210        5220
CTTCCGCCGT TTCTTCCAGG ACAGGTGGAA CCAGCTGGAC CTGGCTATTG TGCTTCTGTC

      5230        5240        5250        5260        5270        5280
CATCATGGGC ATCACACTGG AGGAGATTGA GGTCAATGCT TCGCTGCCCA TCAACCCCAC

      5290        5300        5310        5320        5330        5340
CATCATCCGT ATCATGAGGG TGCTCCGCAT TGCTCGAGTT CTGAAGCTGT TGAAGATGGC

      5350        5360        5370        5380        5390        5400
TGTGGGCATG CGGGCACTGC TGGACACGGT GATGCAGGCC CTGCCCCAGG TGGGGAACCT

      5410        5420        5430        5440        5450        5460
GGGACTTCTC TTCATGTTAT TGTTTTTCAT CTTTGCAGCT CTGGGCGTGG AGCTCTTTGG

      5470        5480        5490        5500        5510        5520
AGACCTGGAG TGTGATGAGA CACACCCTTG TGAGGGCTTG GGTCGGCATG CCACCTTTAG

      5530        5540        5550        5560        5570        5580
GAACTTTGGT ATGGCCTTTC TGACCCTCTT CCGAGTCTCC ACTGGTGACA ACTGGAATGG

      5590        5600        5610        5620        5630        5640
TATTATGAAG GACACCCTCC GGGACTGTGA CCAGGAGTCC ACCTGCTACA ACACTGTCAT

      5650        5660        5670        5680        5690        5700
CTCCCCTATC TACTTTGTGT CCTTCGTGCT GACGGCCCAG TTTGTGCTGG TCAACGTGGT

      5710        5720        5730        5740        5750        5760
CATAGCTGTG CTGATGAAGC ACCTGGAAGA AAGCAACAAA GAGGCCAAGG AGGAGGCCGA

      5770        5780        5790        5800        5810        5820
GCTCGAGGCC GAGCTGGAGC TGGAGATGAA GACGCTCAGC CCGCAGCCCC ACTCCCCGCT

      5830        5840        5850        5860        5870        5880
GGGCAGCCCC TTCCTCTGGC CCGGGGTGGA GGGTGTCAAC AGTCCTGACA GCCCTAAGCC

      5890        5900        5910        5920        5930        5940
TGGGGCTCCA CACACCACTG CCCACATTGG AGCAGCCTCG GGCTTCTCCC TTGAGCACCC

      5950        5960        5970        5980        5990        6000
CACGATGGTA CCCCACCCCG AGGAGGTGCC AGTCCCCCTA GGACCAGACC TGCTGACTGT

      6010        6020        6030        6040        6050        6060
GAGGAAGTCT GGTGTCAGCC GGACGCACTC TCTGCCCAAT GACAGCTACA TGTGCCGCAA

      6070        6080        6090        6100        6110        6120
TGGGAGCACT GCTGAGAGAT CCCTAGGACA CAGGGGCTGG GGGCTCCCCA AAGCCCAGTC

      6130        6140        6150        6160        6170        6180
AGGCTCCATC TTGTCCGTTC ACTCCCAACC AGCAGACACC AGCTGCATCC TACAGCTTCC

      6190        6200        6210        6220        6230        6240
CAAAGATGTG CACTATCTGC TCCAGCCTCA TGGGGCCCCC ACCTGGGGCG CCATCCCTAA

      6250        6260        6270        6280        6290        6300
ACTACCCCCA CCTGGCCGCT CCCCTCTGGC TCAGAGGCCT CTCAGGCGCC AGGCAGCAAT
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DNASIS
T-INS

       6310        6320        6330        6340        6350        6360
AAGGACTGAC  TCCCTGGATG  TGCAGGGCCT  GGGTAGCCGG  GAAGACCTGT  TGTCAGAGGT

       6370        6380        6390        6400        6410        6420
GAGTGGGCCC  TCCTGCCCTC  TGACCCGGTC  CTCATCCTTC  TGGGGCGGGT  CGAGCATCCA

       6430        6440        6450        6460        6470        6480
GGTGCAGCAG  CGTTCCGGCA  TCCAGAGCAA  AGTCTCCAAG  CACATCCGCC  TGCCAGCCCC

       6490        6500        6510        6520        6530        6540
TTGCCCAGGC  CTGGAACCCA  GCTGGGCCAA  GGACCCTCCA  GAGACCAGAA  GCAGCTTAGA

       6550        6560        6570        6580        6590        6600
GCTGGACACG  GAGCTGAGCT  GGATTTCAGG  AGACCTCCTT  CCCAGCAGCC  AGGAAGAACC

       6610        6620        6630        6640        6650        6660
CCTGTCCCCA  CGGGACCTGA  AGAAGTGCTA  CAGTGTAGAG  ACCCAGAGCT  GCAGGCGCAG

       6670        6680        6690        6700        6710        6720
GCCTGGGTCC  TGGCTAGATG  AACAGCGGAG  ACACTCCATT  GCTGTCAGCT  GTCTGGACAG

       6730        6740        6750        6760        6770        6780
CGGCTCCCAA  CCCCGCCTAT  GTCCAAGCCC  CTCAAGCCTC  GGGGGCCAAC  CTCTTGGGGG

       6790        6800        6810        6820        6830        6840
TCCTGGGAGC  CGGCCTAAGA  AAAAACTCAG  CCCACCCAGT  ATCTCTATAG  ACCCCCCGGA

       6850        6860        6870        6880        6890        6900
GAGCCAGGGC  TCTCGGCCCC  CATGCAGTCC  TGGTGTCTGC  CTCAGGAGGA  GGGCGCCGGC

       6910        6920        6930        6940        6950        6960
CAGTGACTCT  AAGGATCCCT  CGGTCTCCAG  CCCCCTTGAC  AGCACGGCTG  CCTCACCCTC

       6970        6980        6990        7000        7010        7020
CCCAAAGAAA  GACACGCTGA  GTCTCTCTGG  TTTGTCTTCT  GACCCAACAG  ACATGGACCC

       7030        7040        7050        7060        7070        7080
CTGAGTCCTA  CCCACTCTCC  CCCATCACCT  TTCTCCACCG  GGTGCAGATC  CTAGCTCCGC

       7090        7100        7110        7120        7130        7140
CTCCTGGGCA  GCGTTTCTGA  AAAGTCCCAC  GTAAGCAGCA  AGCAGCCACG  AGGCACCTCA

       7150        7160        7170        7180        7190        7200
CCTGCCTTCT  TCAGTGGCTG  GTGGGGATGA  CGAGCAGAAC  TTCCGGAGAG  TCGATCTGAA

       7210        7220        7230        7240        7250        7260
GAGAACACAG  CCCTGGAGCC  CCTGCCTCCG  GGAAGAAGGA  AAAGGAGAAA  GCCCAGTGTG

       7270        7280        7290        7300        7310        7320
GCCAAGGCTC  CCGACACCAG  GAGCTG,...  .........  .........  .........
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end of
SEQ 1+3

SEQ ID NO:4

start of SEQ ID NO:2 (coding region)

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E L S * T G P P G D S A S S L E P P T C S P T G V P R L R E D T S S E G L R S P L F G P P G
A P A G Q R ■ D E E E D G A G A E E S G Q P R S F T Q L N D L S G A G G R Q G P G S T E K
D P G S A D S E A E G L P Y P A L A P V V F F Y L S Q D S R P R S W C L R T V C N P W F E
R V S M L V I L L N C V T L G M F R P C E D I A C D S Q R C R I L Q A F D D F I F A F F A V
E M V V K M V A L G I F G K K C Y L G D T W N R L D F F I V I A G M L E Y S L D L Q N V S
F S A V R T V R V L R P L R A I N R V P S M R I L V T L L L D T L P M L G N V L L L C F F V
F F I F G I V G V Q L W A G L L R N R C F L P E N F S L P L S V D L E P Y Y Q T E N E D E S
P F I C S Q P R E N G M R S C R S V P T L R G E G G G G P P C S L D Y E T Y N S S S N T T
C V N W N Q Y Y T N C S A G E H N P F K G A I N F D N I G Y A W I A I F Q V I T L E G W V
D I M Y F V M D A H S F Y N F I Y F I L L I I V G S F F M I N L C L V V I A T Q F S E T K Q R
E S Q L M R E Q R V R F L S N A S T L A S F S E P G S C Y E E L L K Y L V Y I L R K A A R
R L A Q V S R A I G V R A G L L S S P V A R S G Q E P Q P S G S C T R S H R R L S V H H L
V H H H H H H H H Y H L G N G T L R V P R A S P E I Q D R D A N G S R R L M L P P P S T
P T P S G G P P R G A E S V H S F Y H A D C H L E P V R C Q A P P P R C P S E A S G R T V
G S G K V Y P T V H T S P P P E I L K D K A L V E V A P S P G P P T L T S F N I P P G P F S S
M H K L L E T Q S T G A C H S S C K I S S P C S K A D S G A C G P D S C P Y C A R T G A G
E P E S A D H V M P D S D S E A V Y E F T Q D A Q H S D L R D P H S R R R Q R S L G P D A
E P S S V L A F W R L I C D T F R K I V D S K Y F G R G I M I A I L V N T L S M G I E Y H E Q
P E E L T N A L E I S N I V F T S L F A L E M L L K L L V Y G P F G Y I K N P Y N I F D G V I
V V I S V W E I V G Q Q G G G L S V L R T F R L M R V L K L V R F L P A L Q R Q L V V L M
K T M D N V A T F C M L L M L F I F I F S I L G M H L F G C K F A S E R D G D T L P D R K
N F D S L L W A I V T V F Q I L T Q E D W N K V L Y N G M A S T S S W A A L Y F I A L M T
F G N Y V L F N L L V A I L V E G F Q A E E I G K R E D A S G Q L S C I Q L P V N S Q G G D
A T K S E S E P D F F S P S V D G D G D R K K R L A L V A L G E H A E L R K S L L P P L I I
H T A A T P M S L P K S S S T G V G E A L G S G S R R T S S S G S A E P G A A H H E M K S
P P S A R S S P H S P W S A A S S W T S R R S S R N S L G R A P S L K R R S P S G E R R S
L L S G E G Q E S Q D E E E S S E E D R A S P A G S D H R H R G S L E R E A K S S F D L P D
T L Q V P G L H R T A S G R S S A S E H Q D C N G K S A S G R L A R T L R T D D P Q L D G
D D D N D E G N L S K G E R I Q A W V R S R L P A C C R E R D S W S A Y I F P P Q S R F R
L L C H R I I T H K M F D H V V L V I I F L N C I T I A M E R P K I D P H S A E R I F L T L S N
Y I F T A V F L A E M T V K V V A L G W C F G E Q A Y L R S S W N V L D G L L V L I S V I
D I L V S M V S D S G T K I L G M L R V L R L L R T L R P L R V I S R A Q G L K L V V E T L
M S S L K P I G N I V V I C C A F F I I F G I L G V Q L F K G K F F V C Q G E D T R N I T N K
S D C A E A S Y R W V R H K Y N F D N L G Q A L M S L F V L A S K D G W V D I M Y D G L
D A V G V D Q Q P I M N H N P W M L L Y F I S F L L I V A F F V L N M F V G V V V E N F H
K C R Q H Q E E E A R R E E K R L R R L E K K R R N L M L D D V I A S G S S A S A A S
E A Q C K P Y Y S D Y S R F R L L V H H L C T S H Y L D L F I T G V I G L N V V T M A M E
H Y Q Q P Q I L D E A L K I C N Y I F T V I F V F E S V F K L V A F G F R R F F Q D R W N Q
L D L A I V L L S I M G I T L E E I E V N A S L P I N P T I I R I M R V L R I A R V L K L L K M
A V G M R A L L D T V M Q A L P Q V G N L G L L F M L L F F I F A A L G V E L F G D L E C
D E T H P C E G L G R H A T F R N F G M A F L T L F R V S T G D N W N G I M K D T L R D C
D Q E S T C Y N T V I S P I Y F V S F V L T A Q F V L V N V V I A V L M K H L E E S N K E A
K E E A E L E A E L E L E M K T L S P Q P H S P L G S P F L W P G V E G V N S P D S P K P G
A P H T T A H I G A A S G F S L E H P T M V P H P E E V P V P L G P D L L T V R K S G V S R
T H S L P N D S Y M C R N G S T A E R S L G H R G W G L P K A Q S G S I L S V H S Q P A D
T S C I L Q L P K D V H Y L L Q P H G A P T W G A I P K L P P P G R S P L A Q R P L R R Q A
A I R T D S L D V Q G L G S R E D L L S E V S G P S C P L T R S S S F W G G S S I Q V Q Q R
S G I Q S K V S K H I R L P A P C P G L E P S W A K D P P E T R S S L E L D T E L S W I S G
D L L P S S Q E E P L S P R D L K K C Y S V E T Q S C R R R P G S W L D E Q R R H S I A V
S C L D S G S Q P R L C P S P S S L G G Q P L G G P G S R P K K K L S P P S I S I D P P E S Q
G S R P P C S P G V C L R R R A P A S D S K D P S V S S P L D S T A A S P S P K K D T L S L
S G L S S D P T D M D P * V L P T L P H H L S P P G A D P S S A S W A A F L K S P T * A A S
S H 2071 E A P H L P S S V A G G D D E Q N F R R V D L K R T Q P W S P C L R E E G K G E
S P V W P R L P T P G A
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end of SEQ 2 + 4